REAL TIME, LOW COST TECHNOLOGIES FOR DETERMINING TREATED OIL AND GAS PRODUCED WATER STABILITY

A Thesis

by

ALLANA RAE ROBERTSON

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Chair of Committee,	Xingmao "Samuel" Ma
Co-Chair of Committee,	David Burnett
Committee Member,	Bill Batchelor
Head of Department,	Robin Autenrieth

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ABSTRACT

Microbial activity in raw repurposed waters from oil and gas operations is known to cause enhanced corrosion in flow lines and downhole fouling in the reservoir if left un-treated. For this reason, shale operators prefer to use biocides or mobile treatment units to treat raw production waters. Unfortunately, field experience has shown that biocide treatments alone are ineffective and costly. In addition to these findings, produced water stability during storage is not yet well documented due to difficulty in obtaining timely and accurate microbial levels.

Concluding an extensive literature review, industry inquiry, and referencing three levels of scale up testing (laboratory, pilot, and field scales), water treatment studies identified three low cost, real time analysis technologies. Using the identified technologies, membrane filtration was evaluated as a technique to reduce microbial activity and primary microbial metabolites in raw produced waters.

Filtration treatment does efficiently reduce biomass levels in produced water. Using a two-stage filtration scheme with micro and nano filtration membranes, a significant reduction of divalent ion species and of biological activity is observed in permeate waters. Monovalent species were not found to be directly affected by filtration treatment. Metabolically active monovalent ions including: nitrate, ammonia, ammonium and nitrite were found to be reduced by microbial activity in permeate during temporary storage. Additional metabolically active ions including: soluble iron, sulfate, manganese, and dissolved organic carbon were found to be reduced by filtration treatment. Their divalent nature and organic compound molecular weight are thought to

be the source. Concluding the study, it was found that treated produced water still exhibits an unstable, nutrient rich nature capable of supporting microbial growth and oxidation-reduction activity during storage. Therefore, without the addition of a biocide to establish a residual concentration, microbial biomass levels can be expected to regrow.

Current publications available to industry members primarily focus on identifying corrosion and the specific bacteria responsible. Little information is published on methods of treatment and quality control. In addition few “field ready” biological activity monitoring methods are available to the industry. Publishing this paper would provide information about testing technology and the specific metabolic species that must be monitored to ensure efficient microbial mitigation during treatment efforts.

DEDICATION

I dedicate this work to my mother, father, brother, and the rest of my family. Your support and determination has helped me grow into the woman I am today.

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Above all I would like to thank God, through him all things are possible. Looking back, sometimes we wonder why we made the decisions we did, and how these decisions ultimately led us to where we are now. I firmly believe that although some people are more challenging (stubborn), we are all eventually guided to our calling. Therefore, I am grateful that I serve a patient and forgiving God.

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NOMENCLATURE

Acid-producing bacteria (APB) – anaerobic bacteria that form acid during metabolic activity.

Biological Oxygen Demand (BOD) – oxygen required for microbial activity.

Caking- layering of organic matter on the surface of a membrane due to Van der Waals interactions.

Chemical Oxygen Demand (COD) – oxygen required for chemical redox activity.

Downhole – refers to activity within the petroleum reservoir underground.

Concentrate Mode –refers to type of process where the membrane reject stream is recirculated back into the filtration system feed tank. Reject water then becomes recirculated back to the filter for another pass. This was carried out all day to increase the clean water yield and decrease the volume of wastewater.

Extra polymeric substance (EPS) – another term used to identify slime from slime producing bacteria.

General heterotrophic bacteria (GHB) – general aerobic bacteria that grow in aquatic systems. This group also contains nitrifying bacteria capable of oxidizing ammonium and ammonia to nitrate.

Iron-reducing bacteria (IRB) – bacteria capable of reducing iron during metabolic activity.

Iron-oxidizing bacteria (IOB) – bacteria capable of oxidizing iron during metabolic activity.

Methanogens – anaerobic bacteria that forms methane as a metabolic byproduct.

Microbial induced corrosion (MIC) - corrosive action resulting from the production of a bio compound capable of carrying out redox activity at the metallic surface.

Microfiltration (MF) - lowest level of membrane filtration. Pore sizes range from 0.04-4.5 µm. (Appendix A)

Nanofiltration (NF) - the third highest level of membrane filtration. Pore sizes range from 0.008-0.0009 µm. (Appendix A)

Nitrate-reducing bacteria (NRB) – microorganism that utilizes nitrate as the electron acceptor during denitrification.

Reverse Osmosis (RO) - the highest level of membrane filtration. Pore sizes range from 0.002-0.0001µm. (Appendix A)

Slime producing bacteria (SPB) – bacteria capable of excreting extracellular polysaccharide polymers.

Sulfate-reducing bacteria (SRB) –microorganism that utilizes sulfate as terminal electron acceptor in anaerobic respiration.

Sulfite-producing bacteria (SuRB) – form of anaerobic sulfur reducing bacteria that form sulfite during anaerobic respiration.

Thiosulfate-reducing bacteria (TRB) –microorganism which utilizes thiosulfate as terminal electron acceptor in anaerobic respiration.

Total carbon (TC) – total carbon values composed of both total inorganic and total organic values

Total inorganic carbon (TIC) – total carbon in the form of carbonate, bicarbonate, or carbonic acid.

Total organic carbon (TOC) – total carbon composed of organic based materials such as hydrocarbons, humic or fulvic acids. Carbon that is not in the form of carbonate, bicarbonate or carbonic acid.

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CHAPTER I

INTRODUCTION

Statement of the Problem

Growing concerns about water have forced many industries to re-evaluate their water usage. Better water management plans enable companies to reduce capital operating costs while maintaining production. Arid locations currently supporting large oil and gas operations are concerned about water shortages, as a result of population growth. In response, oil and gas is one of the leading industries pushing the adoption of water re-use.

Estimates show that water acquisition, storage, transfer, and disposal can account for approximately10 percent of a well's total cost.[1] In 2007 the average cost to complete a well was approximately 4 million dollars.[2] This average cost includes both conventional and unconventional well development. More recently shale development has dominated the total well completions in the U.S. In 2015, because of the uncertainty in oil and gas markets, companies have been forced to reduce costs severely. Swift Energy, an Eagle Ford Shale operator, reported wells cost down from $3.2 million to $2.2 million.[3] Considering that 10 percent is still a good estimate for water management costs, the total estimate for water management of an oil and gas well would be approximately $200,000 in 2015. Costs for water management include fresh water purchase, water transport, water storage, produced water disposal and the addition of production chemicals (including biocide/corrosion inhibitor costs). Operators understand

the importance of smart management, and they are working to lower these costs for each well site.

Treatment of water with biocide and corrosion inhibitors for drilling and completion activities can cost more than $25,000 per well depending on the quality.[4] Even after treatment, high levels of equipment failures are still encountered in the field. These failures are attributed to the ineffective dosage of biocides and anti-scaling chemicals.[5] Despite the obvious need, general industry standards for the effective use of corrosion inhibition and biological control chemicals do not currently exist for small business owners.

In addition to production company concerns, municipal fears are starting to build. Freshwater used in oil and gas operations in areas under drought conditions is building concern regarding aquifer recharge and sustainability.[1] Due to this, companies have begun promoting produced water re-use and the use of brackish water. The Eagle Ford is currently using approximately 20% brackish water, and the Permian Basin is using approximately 30% brackish water.[1] While promoting produced water re-use and the use of brackish water is expected to ease the stress of water demands, the use of communal water sources increase the probability of contaminated production systems.

Texas, Colorado, and New Mexico government officials have passed laws to protect and promote the use of recycled produced water in the place of fresh water. The Texas House Bill passed on May 22, 2013 states that the person agreeing to take possession of the waste for subsequent beneficial treatment or disposal takes legal responsibility until the waste is transferred to another party for use or disposal.[6]

Colorado's produced water re-use and recycling rule outlines oil well maintenance requirements and points of compliance for wells reusing produced water.[7] New Mexico's new oil and gas produced water re-use rule provides requirements for the storage, re-use, use of recycling facilities, recycling of contaminants, and requirements for the protection of fresh water, public health, and the environment.[8]

Commercial services for increased reuse of produced water are already available in order to comply with new regulations.[1] Mobile treatment units built to treat produced water at well site locations are already being used. Systems include a range of the following technologies: thermal desalination, electrocoagulation pared with chlorine dioxide, processes pared with dissolved air flotation, membrane filtration pared with on-site chemical oxidation, and mobile clarification systems.[1]

Microfiltration and nanofiltration technologies have been observed to have the most appropriate low energy demand, and small environmental footprint for produced water treatment tests. High levels of dissolved organics, salts, solids, and biological components make treating this type of water with membrane filtration a challenge. Addressing this problem, technology developers are evaluating the application of a more aggressive pretreatment, which will make chemical biocide and anti-scaling treatments more efficient and cost effective.

Pre-treatment procedures are generally focused on removing hydrocarbons, total suspended solids and reducing dissolved scaling ion concenrations.[9] Bag and cartridge filters connected in sequence with oil coalescing filters are generally used in most pre-treatment procedures to accomplish removal. Many studies document success with the

employment of stricter pretreatment procedures in treatment schemes.[9] Monitoring bacterial activity however, has not been identified as an important water component to monitor. This study is designed to address this issue.

Measurement and Control of Microbial Activity in Produced Water

Microbial activity has often been overlooked when carrying out water quality analysis on produced waters intended for reuse. Chemical metabolites and ions present in high concentrations in produced brines promote microbial growth. As a result, a higher incidence of microbial induced corrosion (MIC) has been observed at well sites reusing water. Current microbial techniques do not support real time analysis for reporting quick and accurate results. Instead, operators must wait for traditional plate count methods to incubate over several days. During this time, critical action to mitigate MIC is overlooked in operations. Adding to the confusion, general standards do not exist for companies to follow to ensure treated water quality exhibits reduced corrosion rates.

Approach to Solve the Problem

The purpose of this study therefore, is to evaluate the use of real time microbial analysis technology in quantifying microbial activity in membrane treated produced waters. Chemical components related to microbial growth and biological activity will also be monitored to determine if membrane treated water could still exhibit bacterial growth during storage or use. Results of the study will be used to plan future field trials of produced water treatment by A&M researchers.

Research Objectives

The specific objectives of this study are:

1. Determine water stability when treating continuously
    i. Microbial activity post continuous treatment
    ii. Reduction of microbial electron donors and acceptors
    iii. Reduction of dissolved organic and inorganic carbon
    iv. Total hardness reduction
    v. Microbial nutrient levels post filtration treatment with MF and NF systems
2. Determine microbial water stability during suspended treatment
    i. Equipment failure
    ii. Storage in open and sealed containment

Significance of the Study

Following the completion of this study, a broader overview of water quality will be established for untreated and treated produced water samples. Open access to data will ensure that small and large businesses can utilize information for optimizing current water treatment plans. Ultimately, the goal of this study is to create a deeper understanding of both chemical and biological components that cause biological activity in produced water. Treatment of water sources with methods similar to the described should be optimized to enhance treatment efficiency and promote environmental awareness.

Theoretical Framework

Membrane dynamics must be considered when understanding the mechanisms involved in the separation of bacteria, dissolved organic matter, and ionic species. Since produced water contains high concentrations of ionic species, precipitation is more likely to occur during treatment. However, dissolved organic complexation reactions with heavy metal ions can prevent the removal of ionic species and bacteria from the water column.

Calcite, barite, celestite, anhydrite, gypsum, iron sulfide, and halite are considered the top seven precipitants commonly observed in oil and gas produced waters.[10, 11] Barite and $CaCO_3$ homologs are expected to be the dominant species present in produced waters.[11] Dissolved organic matter on the other hand, is likely to contain components of crude oil as well as humic and fulvic acids. Dissolved organic matter can exist in different molecular configurations while in water.[12]

Crude oil is generally characterized as containing paraffins, napthenes, aromatics, and asphaltics.[13] General crude composition is expected to resemble the following percentages: 15-60% paraffins, 30-60% napthenes, 3-30% aromatics and the remaining percentage asphaltics.[13, 14] The produced water used in both experimental studies is expected to contain residual Eagle Ford crude. Eagle Ford crude is generally expected to exhibit 85% napthenes, and 15% aromatics and asphaltics.[15] Napthenes, paraffins, and aromatics are expected to be largely stable and unreactive in raw crudes.[13] However, asphaltics are expected to contain porphyrins, specifically etioporphyrins and dexophylleorithroetioporphyrin (DPEP).[16] These hydrocarbon metal complexes can be

structured in a manner resembling chlorophyll and heme complexes.[16] Asphaltics are therefore expected to represent the reactive hydrocarbon species that could play a crucial role in species precipitation.

Humic and fulvic substances are complex compounds consisting of condensed aromatic nuclei with –OH and –COOH groups.[17] Their structures can resemble chlorophyll or heme complexes as well however, both humic and fulvic substances are much larger in size and structure. Although their exact structure is not known, fulvic substances are known to be smaller than humic substances with twice the oxygen content.[18] Fulvic substance oxygen content is also found in the form of –OH and –COOH groups.[18] Fulvic substances are considered to be more reactive than humic substances making them more likely to complex with metal ions.[17, 18]

Metal ion complexed organic particles and bacteria will be expected to move toward the membrane surface by convective diffusion. Organic adsorption to accumulated particles at the membrane surface is thought to reduce the amount of organics available to directly adsorb to the membrane.[19] The layering effect, caking, that occurs as a result of this action reduces the flow through the membrane, and increases the power consumption needed to maintain the permeate flow rate.[20, 21] Therefore, the already compacted membrane surface is forced to compact even further as the pressure is increased to maintain the permeate flow. Unfortunately, additional organics, bacteria and precipitant colloidal particles will be forced onto the membrane surface and into the inner pores further reducing the permeate flow rate as the pressure is increased.[20, 21] Delayed chemical cleaning of membranes will result in microbial damage that will

permanently reduce the membrane's operational efficiency.[20] Fouling as described is inevitable when treating produced brines.[19]

Charged membranes behave in a similar manner as described above however, charge repulsions and pH changes can have larger effects on membrane rejection. Sulfonic acid functional groups present on the NF membrane surface and within the membrane matrix create an overall negative charge.[22] This negative charge is thought to be the underlying mechanism responsible for NF separation. Steric hindrance (sieving affect) and electrostatic effects (Donnan effect) resulting from the negative charges of the membrane matrix slow the movement of small molecules and ions through the membrane, and prevent the passage of larger macromolecules.[20]

Low pH and high ionic strength oil and gas waters are expected to reduce charge repulsions both at the surface and within the membrane matrix.[22] Reduced charge repulsions enable the membrane to shift into a more compact state reducing permeate flux.[22, 23] Accumulation of precipitated species, colloidal particles, and dissolved organics at the surface is thought to be responsible for the increased shielding and overall fouling potential.[22] Colloidal fouling can result from deep penetration of particles into the inner membrane and the build-up of a cake on the membrane surface.[21]

All interactions described above must be considered when evaluating membrane separations for oil and gas produced waters. Produced water is a very complex aqueous phase that contains both microbial, organic and ionic complexes. Olatubi et al. studies these phenomena extensively.[24] Considering this information, it can be assumed that un-associated monovalent ions, will likely pass through the caking layer of microfiltration

(MF) and NF membranes, but will be rejected from reverse osmosis (RO) membranes.[25] Un-associated divalent ions, slightly larger ions, will pass through the caking layer of MF membranes, but will be rejected from NF and RO membranes.[25-27] Since bacterial particulate matter is significantly larger than ionic components, it can be expected that MF, NF, and RO will all reject this type of particulate matter.[28, 29] MF membranes however, are more widely used for solids removal to protect NF and RO systems downstream. For additional information on membrane rejection capabilities, please see the Osmonics filtration spectrum (Appendix A).

According to our understanding of membrane mechanics, microbial, dissolved solids, and divalent ion species should be removed from the permeate. Storage and transport of water therefore, should be considered to reduce the effects of opportunistic environmental bacteria that may contaminate treated water prior to reuse.

Assumptions

- Microfiltration and nanofiltration units were operated to achieve stable pressure and flow rate conditions prior to sample collection.
- Membrane flux effects were minimized by small changes in the feed pressure and flow rate.
- Lost process time is the most important component during an equipment failure.
- Microbial group identification could be carried out based on nutrient concentrations in raw produced water.

Limitations of the Study

- This study was designed only for oil and gas hydraulic fracturing operations.
- The data may not be generalized to other treatment processes.
- The study results may not be used to identify specific bacterial species.
- The study results do not establish microbial nutrient concentration standards however, the results can be used as an aid to establish such standards.

CHAPTER II

REVIEW OF LITERATURE*

Treatment processes are often specific to individual service companies, and releasing information about treatment procedures is viewed as releasing proprietary information. As a result, documents assessing these procedures are rare. However there have been many studies carried out outlining corrosion and operational issues in the field as a result of water reuse. Current technologies that can be used to carry out field analysis for real time results are also evaluated in some of these studies. Eboagwu and Beech serve as examples of some of this published work.[30, 31] Both provided foundational knowledge in their thesis work with GPRI for the use of membrane filtration to treat produced waters prior to reuse.

Overview of Microbial Activity in Oil Field Waters-as Stated in Power Across Texas Collegiate Team Report

Microbial activity has been overlooked in the past by corrosion experts as a possible contributor to system corrosion. However, as a result of the molecular technology boom, oil field microbiologists have begun identifying microbes present in industrial system biofilms. Molecular techniques offer a more accurate quantification

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and identification of the microbes in a sample as opposed to the traditional culture based methods. Culture methods are slow, and rely mainly on the ability of the scientist to replicate all of the growth conditions.

This is quite a challenge as bacterial communities present in biofilms vary according to the environmental conditions of the produced water. Ten broad groups have been identified from produced water studies as key players in MIC. The ten are as follows: Sulfate-reducing bacteria (SRB), thiosulfate-reducing bacteria (TRB), nitrate-reducing bacteria (NRB), sulfite-producing bacteria (SuRB), acid-producing bacteria (APB), methanogens, slime producing bacteria (SPB) also categorized as extra polymeric substance (EPS) producing bacteria, iron-reducing bacteria (IRB), general heterotrophic bacteria (GHB), and iron-oxidizing bacteria (IOB).[33, 34] It is important to note that one bacterial group alone is not solely responsible for MIC.[33] The establishment of a matrix of extracellular polymeric substances on existing corrosion deposits creates a sticky web like environment for other bacterial species in the water column.[34] Layers form as the biofilm grows, creating a protective layer for the species closest to the metal surface. Treatments with biocides often fail to reduce MIC activity because of the layering system. Biocides can diffuse into the biofilm only a short distance before the gradient potential is lost. As a result, oil field equipment experiences localized corrosion through pitting and sulfide stress cracking as result of acid, H_2S and FeS production through metabolic activity of microbes.[34]

Oil and gas operators and service providers constantly encounter and combat bacterially related issues and are looking at new molecular based techniques for

assistance in making smarter decisions for bacterial control programs. AES Water Solutions, an example of a service provider, understands the industry's need for better microbial monitoring. AES offers an alternative MIC management plan combining molecular benchtop methods with a non-chemical biocidal treatment.[35] In late December 2013 a coil tubing failure was experienced in the Eagle Ford Shale after a 2-3/8" (60.3mm) 100 grade string broke during milling operations.[34] The failure was attributed to recycled water being circulated through the tubing at the time of the break from previous stimulation operations.[34] Metagenomic analysis was carried out to quantify the amount of bacteria present in the system at the time of the break as well as in the source water.[34] Results yielded a high level of bacteria and a large variety of species in the source water. The tubing appeared to exhibit a brittle fracture and heavy corrosion on the inside walls of the tubing. Company mitigation management details were not given in the report. Corrosion was attributed to bacterial activity in the recycled water as the tubing was never used to pump concentrated acids.[34] According to this case study, coil tubing appears to be more susceptible to MIC corrosion than standard pipe when using recycled and grey municipal water. Time delays were also mentioned as a negative impact on corrosion and MIC management systems implemented by companies. Commercial lab results take at least 2-3 days to receive from the time of sampling. During this time, the operator is unaware of the effect of the biocide dosage on the microbial population in the produced water, and is unable to determine the biocide efficiency. Conference feedback relayed that multiple coil tubing service providers are beginning to see the importance of monitoring MIC, and how it can financially affect

their operations post-failure.[34] The need for quick and accurate tests at the field site is starting to be realized by industry members.

In addition to corrosion, microbes can also plug fractured zones within a producing well. Insoluble biomass, biopolymers, and biominerals produced by microbial activity have been observed to reduce flow in areas with high water permeability.[36, 37] Reduced flow means that a producing well now produces at a slower rate because oil and water must now diffuse around the plugged zones toward the point of injection which now serves as the point of exit.

Plugged bacteria are now lodged within the reservoir in a nutrient rich area that is not feasible to treat with biocide. Reservoirs often contain high concentrations of sulfates, chlorides, and various electron donor species microbes, specifically SRB, can use to generate hydrogen sulfide (H_2S) gas. The production of hydrogen sulfide gas is often referred to as souring. Sour water, reservoir souring, and sour gas all describe the production of hydrogen sulfide in various mediums throughout the oil and gas industry. Sour water and sour gas produce health and safety issues for oil and gas workers as well as community members in areas surrounding production sites. The presence of hydrogen sulfide increases the overall cost companies must spend on outfitting their employees with the appropriate safety equipment. Hydrogen sulfide gas also increases the environmental laws and regulations companies must follow, or pay after breaking stated emission levels.

Using a reservoir modeling study based on an oilfield in Japan, the rate of microbial growth from the point of injection can be observed. Bacterial growth was

determined by carrying out a kinetic analysis based on the estimated concentration of sulfate, ethanol, and the temperature of the reservoir near the injection site.[38] SRB growth was observed to be highest near the injection site where the temperature was lower than 50°C.[38] Although mesophilic SRB were mainly observed in the study (SRB that grow below 50°C), thermophilic SRB was also mentioned as a possible species that could exhibit growth above 50°C.[38] Expanding the growth temperature increases the area of the reservoir that can be affected by SRB populations. As a result of the study, thorough treatment of injection water sources was highly recommended to negate reservoir plugging and souring. Even low amounts of SRB bacteria have been identified to cause hydrogen sulfide gas generation over production life of the well. However, due to limitations of current treatment technologies, reducing the microbial populations in injection water is the only economically feasible method for the oil and gas industry at this time.

Water Quality Testing Parameters

Basic water quality parameters were selected to aid in determining if produced water can support microbial activity and if water is suitable for membrane filtration. Most oil and gas field operators consider water quality to encompass ion concentration data as well as the data listed in Table 1 below. However, for the purpose of this study we will consider water quality to encompass only the basic components: temperature, pH, dissolved oxygen, turbidity and alkalinity. Calculations can be performed using this baseline water chemistry data to estimate select component concentrations if needed.

Environmental engineers typically relate increased dissolved oxygen content with water quality improvement. High dissolved oxygen levels indicate lower biological oxygen demand (BOD) and chemical oxygen demand (COD) levels.[29, 12] Reduced BOD and COD levels, for oil and gas waters, implies lower levels of microbial and chemical activities.[29, 12] Table 1 shown below illustrates the importance of each baseline water quality parameter.

Table 1. Baseline Water Quality Components for Characterization

Basic Water Components	Why Chosen	Method of Testing
Temperature	Snap shot of conditions for dissolved components	**Thermocouple**
pH	Provides basic understanding of water chemistry [39]	HACH Probe; **Commercial Lab**
Dissolved Oxygen	Represents one of the most important electron acceptors and will compete with nitrate, sulfate for electrons [39]	**Fischer Scientific Probe**
Turbidity	Needed for NF membrane system, turbidity has to be low enough not to foul NF [40]	**HACH Meter**
Alkalinity	Can help determine HCO3; CO3; OH components for water chemistry [39, 41]	**Commercial Lab**

Current Testing Parameters for Microbial Growth

Testing parameters used by Fichter and An identify sulfate, sulfide, nitrate, nitrite, phosphate, dissolved organic carbon, and ammonia as key microbial nutrients.[42, 43] In an effort to verify the importance of the listed components, literature was analyzed from several well-known microbiology journals. Examples of these journals include: The Journal of Bacteriology, Soil Biology & Biochemistry, FEMS Microbiology Ecology, American Society for Microbiology, and The Journal of Biological Chemistry

to name a few. The following tables were constructed to illustrate the importance of each component to the metabolic activity of microbes.

Table 2. Substrate Components for Water Characterization

Microbial Substrate Components	Mechanism of Action	Method of Testing
Total Iron (Fe^{+3}; Fe^{+2})	Fe^{+3} can be used as electron acceptor in nitrification and general hetero/autotrophic species [39, 44, 45] Fe^{+2} can be used as electron donor in denitrification and acidophiles (some species form acid), Fe^{+2} is oxidized at low pH to $Fe(OH)_3$ [39,41]	HACH Kit**; Commercial Lab**
Manganese	Mn^{2+} can be used as electron acceptor by many bacteria that carry out nitrification, denitrification, and acidophiles (some species form acid) [41, 44,46,47]	HACH Kit; **Commercial Lab**
Total Phosphate (orthophosphate, condensed phosphate, and organic phosphate)	Elevated inorganic and organic phosphorus levels cause microbial and algal growth [41, 48]	HACH Kit**; Commercial Lab**
Sulfate	Used in sulfate reduction as an electron acceptor; Sulfate reducing bacteria [39, 41, 49] Formed from sulfur oxidation by sulfur oxidizing bacteria [49]	HACH Kit**; Commercial Lab**
Thiosulfate	Used in sulfate and H_2S formation by sulfate reducing bacteria [49] Used by sulfur oxidizing bacteria [49]	**Commercial Lab Only**
Sulfite	Used in sulfate and H2S formation by sulfate reducing bacteria [49] Used by sulfate oxidizing bacteria [49]	**Commercial Lab Only**
Sulfide	Used by sulfide oxidizing bacteria to form sulfate [49]	HACH Kit; **Commercial Lab**
Total Nitrogen (nitrite, ammonia, ammonium, nitrate)	Ammonia, ammonium, nitrite, and nitrate used in nitrification [44, 47] Nitrate, and nitrite used in denitrification [39, 44, 45] Nitrate, nitrite, and ammonia used in anaerobic ammonium oxidation[44]	HACH Kit; **Commercial Lab**
TC/TOC/IC	Carbon source for microbial activity [39, 45]	TOC Analyzer**; Commercial Lab**

Table 2 verifies the importance of each key metabolite listed by demonstrating the mechanism of action each component is involved in during cellular metabolism. Each component serves as either an electron donor, electron acceptor, nitrogen source, or carbon source for microbial metabolic action. Table 3 displays the importance of additional ions that are involved in microbial homeostasis. Through adaptation and mutation, microbes are able to sustain a homeostatic environment that allows normal metabolic activity and microbial amplification.

Table 3. Ion Components for Water Characterization

Ion Water Components	Mechanism of Action	Method of Testing
Chloride	Estimate chloride component of salts; chloride ion participates in ion channels and pumps, osmotic regulation/homeostasis [50]	HACH Probe; **Commercial Lab**
Sodium	Participates in ion channels and pumps, osmotic regulation/homeostasis [51]	HACH Probe; **Commercial Lab**
Calcium	Causes water hardness; transported via ion channels and pumps, osmotic regulation/homeostasis [52]	HACH Kit**; Commercial Lab**
Magnesium	Causes water hardness; transported via ion channels and pumps, osmotic regulation/homeostasis [53]	HACH Kit**; Commercial Lab**
Potassium	Participates in ion channels and pumps, osmotic regulation/homeostasis [50, 54]	HACH Kit**; Commercial Lab**
Conductivity	Measures total dissolved ions	HACH Kit; **Commercial Lab**

The ions listed in Table 3 were listed in addition to the key metabolites verified in Table 2 due to their importance in microbial homeostasis. Although disruption of homeostasis is not feasible in aerobic systems, frack ponds and open air storage tanks,

ion concentrations must still be considered to evaluate microbial populations under anaerobic conditions commonly found downhole and in closed storage tanks. Changes in sodium, chloride, calcium, magnesium, and potassium can cause microbial populations to shift due to inhibitory action experienced by select microbial communities unable to grow in highly concentrated systems.[55, 56] Inhibitory action is more prominently observed in anaerobic systems where metabolic fermentation relies on cationic and anionic components to kinetically favor cell synthesis and cell maintenance activities.[12] Please note that volatile organic acids and short chain alcohols can be used to identify fermentative microbial action in water systems.[29, 43]

Shifting from an oxygen rich to an oxygen poor system can also cause changes in microbial populations.[29]

Field Ready, Real-Time Microbial Analysis Technology

Molecular biology has made significant advances since the early 1950's when James Watson, and Francis Crick, identified the double helix structure of DNA. Previous research carried out by Friedrich Miescher, Phoebus Levene, Erwin Chargaff, and Rosalind Franklin played a major role in establishing the scientific foundation key to Watson and Crick's success.[57] Today, similar ground breaking advances are occurring across many industries as microbial activity continues to cause processing upsets and health issues.

The biomedical industry for example, is currently the largest user of molecular tools capable of identifying specific bacterial activity. Biomedical use primarily focuses

on the application of molecular tools for proteomics, stem cell therapy, gene therapy, pharmacogenomics, and the identification of infections.[58] Driving the advancement of technology in the biomedical industry are large pharmaceutical companies or "Big Pharma" capable of supporting the necessary cash flow.[58]

Oil and Gas, like the biomedical field, also uses molecular tools in corrosion identification and process management strategies.[5, 43] The largest difference between the two industries is the need for low cost, mobile treatment units capable of operation at field sites. Real-time analysis at field sites allow engineers and operators to make changes in operations without the financial cost of time delays. Currently, only three mobile technologies have been identified as low cost and field ready. The remaining parts of this section will now look at the three technologies and the published data that supports their use in the field. Unfortunately, only one technology of the three discussed is capable of identifying specific bacterial groups. The remaining two technologies focus on identifying non-specific bacterial activity or biomass. Please note that benchtop technologies such as: general fluorescent microscopy, hydrogenase measurement, respirometry, and fluorescent antibody microscopy can be used in bacterial enumeration too.[59] However, these technologies require a laboratory setting and trained technicians to carryout analysis. Therefore, these technologies will not be discussed in detail due to the study focus on mobile field analysis. The technologies discussed below would require minimum training and could be used by any field worker.

ATP Assay

The ATP assay is the most well-known mobile field technology available to industry. The National Association of Corrosion Engineers, NACE, adopted the ATP assay as a standard for monitoring microbial growth in the oilfield.[59] ATP analysis has been used to evaluate biocide effectiveness, analyze biofilm kinetics, and quantify general microbial activity in oilfield systems.[60-63]

Assay mechanics involve the lysing of cells to free cellular ATP for use in enzymatic catalysis of luciferin to oxyluciferin via the firefly luciferase enzyme.[64, 65] Oxyluciferin can emit light at 560 nm and is therefore detectable by a handheld photometer.[64, 65] The measured intensity can then be used to calculate the mass of active microbes in your sample based on an assumed measure of ATP per cell. LuminUltra sells a version of the ATP assay, and states on their website that their software assumes one cell contains one femtogram (fg) of ATP.[66] ATP is also assumed to rapidly degrade in the environment and will not misidentify dead cells as active microbes.[63, 66] During use in the field, a slight lag in ATP degradation was experienced while carrying out a biocide study in April 2015. Still, the assay reported a consistent trend based on the secondary instrumentation used to analyze microbial activity. Based on this information it can be safely assumed that the ATP assay is very efficient in identifying microbial activity with the exception of delayed fluorescence reduction during kill studies.

Bactiquant-water Meter

Mycometer's Bactiquant-water Meter is a relatively new technology to the U.S. This technology is developed and sold by a Danish biotechnology company, and has been used in studies in both the global food and beverage industry as well as Denmark's municipal industry.[67-70] Bactiquant has been praised as an efficient tool to carry out rapid microbial analysis on site by the USA Water Research Foundation and is currently recommended in their toolbox for potable water monitoring.[70] The Bactiquant-water Meter is also the only total bacteria technology verified by the U.S. Environmental Protection Agency (EPA).[67]

U.S. EPA verification was achieved in 2012 with the publication of a verification report prepared by Battelle.[71] Verification involved a detailed analysis of data linearity, repeatability, inter-assay reproducibility, and data completeness.[71] Bactiquant results were found to be repeatable, and reproducible in the study after analyzing a quality control *Pseudomonas aeruginosa* strain and indigenous bacteria from lake water with four different concentrations and two different analysts to carry out testing.[71]

Mechanism of action involves the use of 0.2 micron filter to trap microbial solid particles. After collection of microbial particles, substrate bound to a fluorophore molecule is pushed through the filter where it reacts with the microbe's hydrolase enzyme.[70] Reaction time can range from 10-30 minutes based on time requirements. Upon completing the reaction, neutralizing solution is pushed through the filter to wash the free fluorophore molecules into the collection cuvette below the filter. The fluorometer is then used to determine fluorescence intensity in fluorescence units (fu).[71]

Once fluoresce intensity is obtained, the value is then inputted into a preprogrammed excel sheet that calculates the Bactiquant value (BQV) or microbial activity for the sample. The number one challenge with this technology is the lack of units comparable to common testing methods such as colony forming units (CFU). Mycometer understands this problem and is working to define an appropriate conversion that will enable Bactiquant data to be reported as CFU's.

Sulfate Reducing Bacteria Detection

SRB detection employs the use of the NACE approved APS-Reductase enzyme specific to SRB bacteria.[59] APS stands for adenosine 5-phosphosulfate. APS-reductase is able to catalyze the reduction of adenosine 5-phosphosulfate to sulfite and adenosine monophosphate.[72] Mobile test kits have been developed based on this knowledge after its publication by Horacek in 1992 in the SPE Drilling Engineering Journal.[72] Horacek's publication was based on the foundational knowledge from Tatnall's testing methods published in 1991 and Horacek's own joint publication with Gawell in 1988 showcasing a new test kit for rapid detection at the SPE Annual Technical Conference and Exhibition in Houston, TX.[72, 73] APS-reductase is thought to be involved in only respiratory sulfate reduction and therefore serves as the terminal electron acceptor in anaerobic respiration.[72] APS-reductase is not involved in assimilatory sulfate reduction and is not found in non-SRB species.[72] Due to this reason, it is assumed that APS-reductase exhibits sufficient specificity to SRB bacteria and would serve as a good selection parameter for SRB identification.

An example of a current mobile testing kit employing this methodology is Modern Water's QuickChek SRB immunoassay detection system. The QuickChek SRB system utilizes the use of purified antibodies to detect the APS-reductase enzyme in both living and non-living SRB cells.[74, 75] Testing methodology involves the capture of SRB particles on a filter and the subsequent lysing to release the APS enzyme.[75] Once lysed, a solution containing the immunoreagent is mixed with the free APS enzyme and allowed to react. The reacted solution is then poured onto a test membrane device and washed with a chromagen liquid for color development.[75] Once the color has been developed, the membrane color is matched with an indicator card to determine SRB concentration.

Modern Water's methodology is very similar to that published by Horacek in 1992. SRB methodology was verified in 1992 by Horacek when field testing determined the SRB test required a maximum of 20 minutes and was able to quantify SRB bacteria not detected by traditional media methods.[72] U.S. EPA verification and quality control reports for current operations were not found during the literature search.

Background Studies

Encana Oil & Gas (USA) Inc. Permian Basin Microbial Study

High corrosion rates in producing wells have pressured Encana Oil & Gas (USA) Inc. to investigate current water management procedures to mitigate further damages. Water is involved in both the drilling and completions processes from day one of site operations. Figure 1 below displays the microbial activity observed in the cores of six wells during drilling operations.

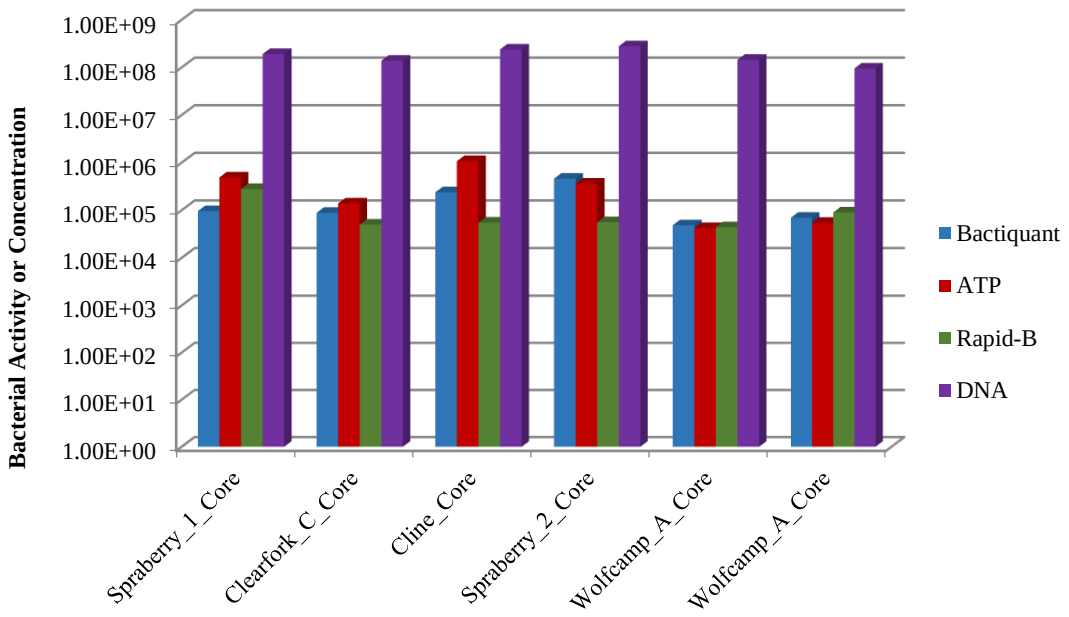

Figure 1. Microbial analysis of core samples during drilling operations for six different well locations on Encana Oil & Gas (USA) Inc. owned fields in Midland, TX. Microbial analysis was carried out with four independent monitoring technologies: Mycometer's Bactiquant-water Meter, LuminUltra's ATP Assay, Vivione Biosciences Rapid-B Flow Cytometer, DNA extraction and quantification carried out by Ecolyse. Each microbial quantification test reports data in independent units. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall bacterial activity or concentration and the individual units neglected. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

A recently acquired oil and gas field was found to exhibit classic signs of a heavy microbial burden, including incidences of hydrogen sulfide production, down hole and surface microbially influenced corrosion, downhole pump and surface equipment fouling and fracturing fluid and drilling mud degradation. Over 130 samples, including formation core material, drilling muds, fracturing fluid source waters, production well samples, samples collected from failed pipe surfaces and samples from salt water disposal facilities, were collected to perform a comprehensive microbial survey.

According to the information provided in Figure 1, microbial contamination is already present as observed from the core samples taken during the drilling process. Prior to core sampling, the use of drilling mud mixed with untreated water to carry rock cuttings to the surface for disposal is assumed to contribute to formation souring. Data provided in Figure 2 supports the assumption of microbial contamination through the use of drilling muds mixed with untreated waters.

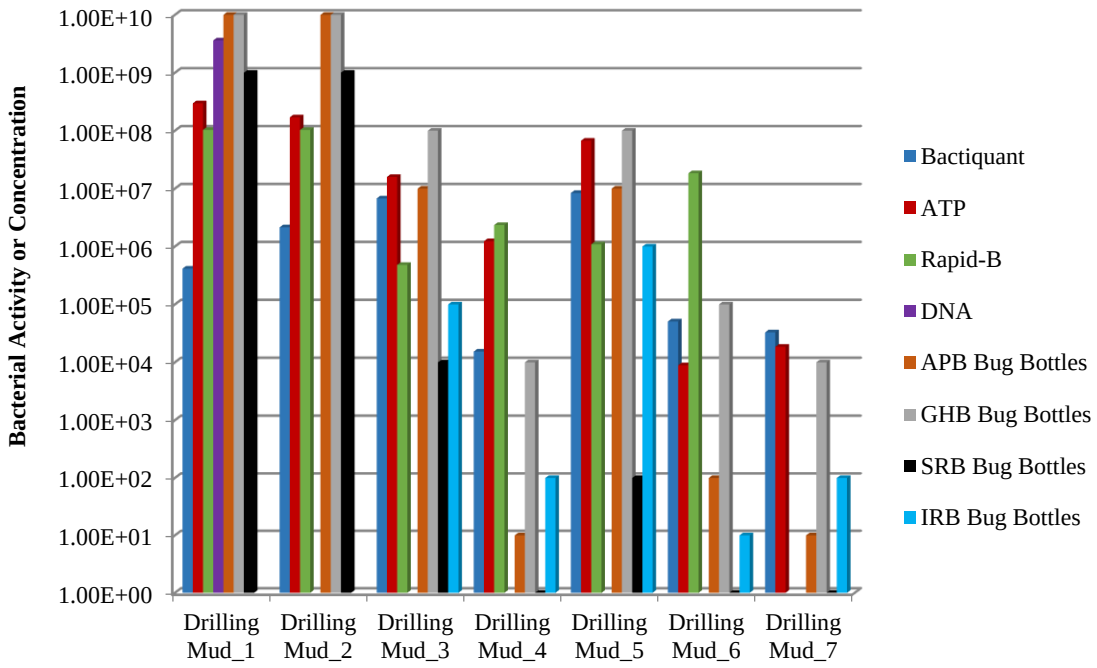

Figure 2. Displays the microbial activity or concentration in drilling muds used in the drilling of four different wells. Difference in oxygen exposures for all seven drilling mud samples is understood to cause a change in microbial activity within the mud sample. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall bacterial activity or concentration and the individual units neglected. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

Microbial activity was also monitored in wells undergoing completion activity. “Completion” is another term in the oil field that defines all necessary field work to carry out hydraulic fracturing of horizontal or vertically drilled wells. Figure 3 below

displays microbial activity in fourteen different hydraulic fracturing water storage pits and one water distribution station.

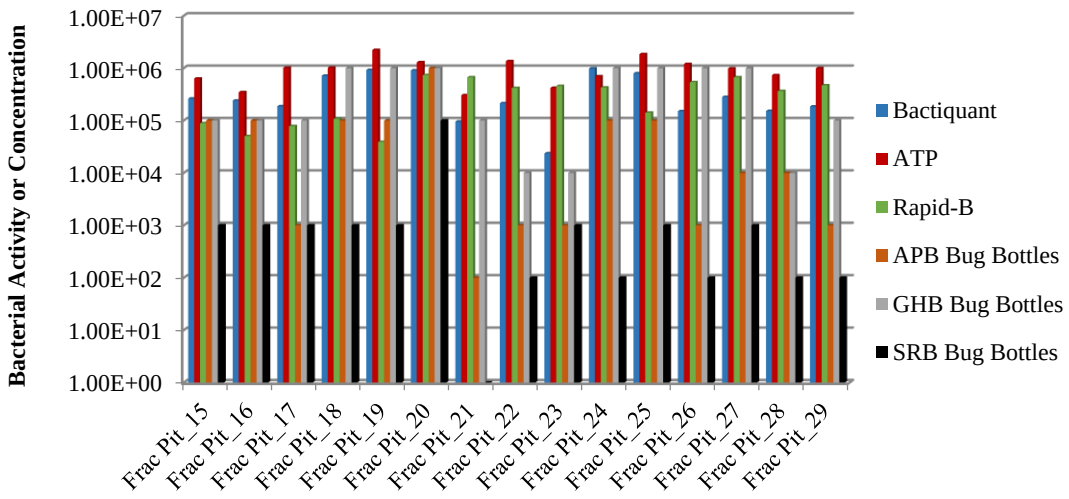

Figure 3. Illustrates the microbial activity or concentration observed in waters stored in open air hydraulic fracturing pits and transported through oil and gas purposed water distribution networks. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall microbial activity or concentration and the individual units neglected. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

Varied populations of microbial activity likely occur due to the layering action that creates anoxic and oxygen poor zones in stagnant water near the bottom of the pond. Oxygen diffusion into stagnant water is limited by the microbial activity in and below the water layer near the pond surface. Water stored in the hydraulic fracturing pits and distributed through the water station listed above will be directly pumped downhole and used in fracturing operations. The level of bacteria displayed above will likely play a key role in future corrosion and souring issues in the wells completed with the above water

sources. Additional hydraulic fracturing information is listed in a secondary graph in (Appendix B).

Production wells that had exhibited potential microbially related operational issues were analyzed to determine severity of microbial contamination. Figure 4 illustrates microbial activity and populations found in twenty two wells with historical bacterial issues.

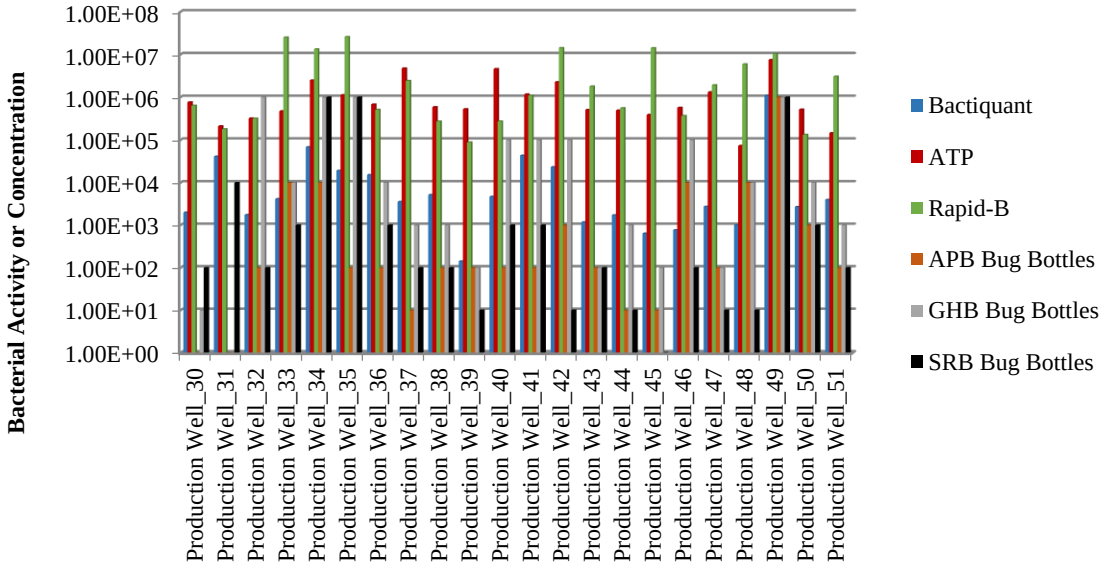

Figure 4. Displays microbial activity in twenty two production wells with historical bacterial issues. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall microbial activity or concentration and the individual units neglected. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

High levels of APB, GHB, and SRB microbes lead to well souring. Production wells listed above were likely completed with water sources also experiencing high levels of microbial activity prior to completion. Mixing water sources already containing microbial activity with nutrient rich additives for completion activities as well as

additional water from reuse efforts causes the results in Figure 4. Additional production well information is listed in a secondary graph in (Appendix B).

Production water from reuse and production operations is disposed of in salt water disposal wells (SWD's). Because the salt water disposal wells are designed for long-term water disposal, it is important that bacterial contamination, suspended solids and oil carryover are closely monitored to ensure well sustainability. Figure 5 below illustrates microbial activity in Encana Oil & Gas (USA) Inc. SWD wells.

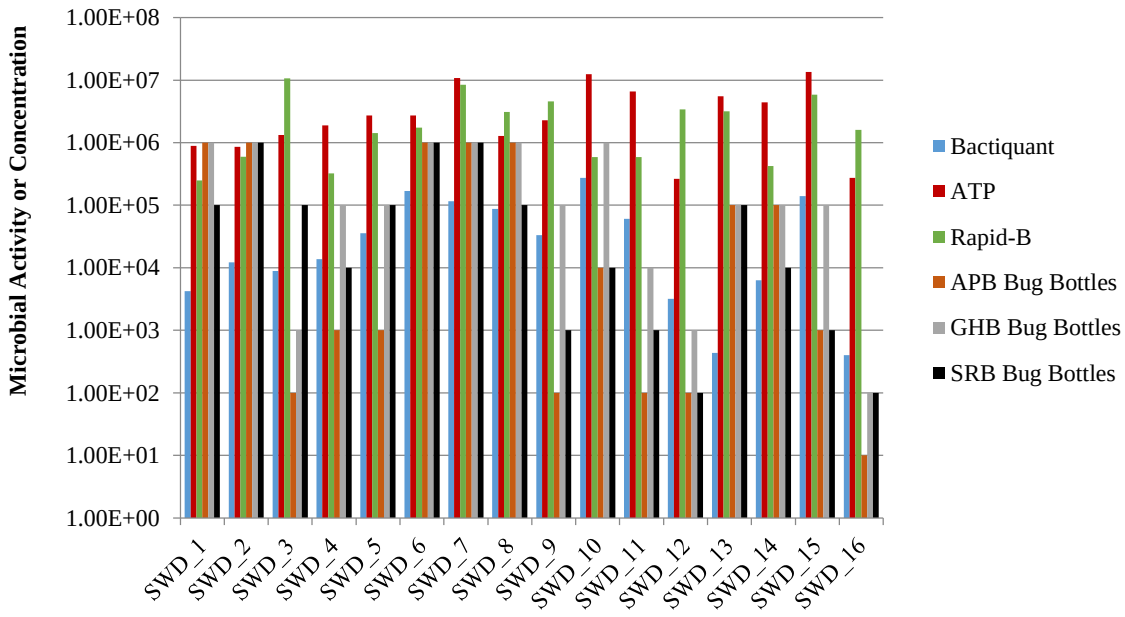

Figure 5. Illustrates microbial activity in select Encana Oil & Gas (USA) Inc. SWD wells in the Midland, TX area. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall microbial activity or concentration and the individual units neglected. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

The purpose of this graph is to show that SWD water is a highly concentrated and microbial active water handled by upstream oil and gas operations. Filtration treatment is

thought to be well tested under the conditions provided by SWD waters. According to this reasoning SWD water was selected as the water for use during experimental membrane filtration treatment.

Summarizing the findings of the Encana Oil & Gas (USA) Inc.'s Microbial Study is Figure 6 provided below.

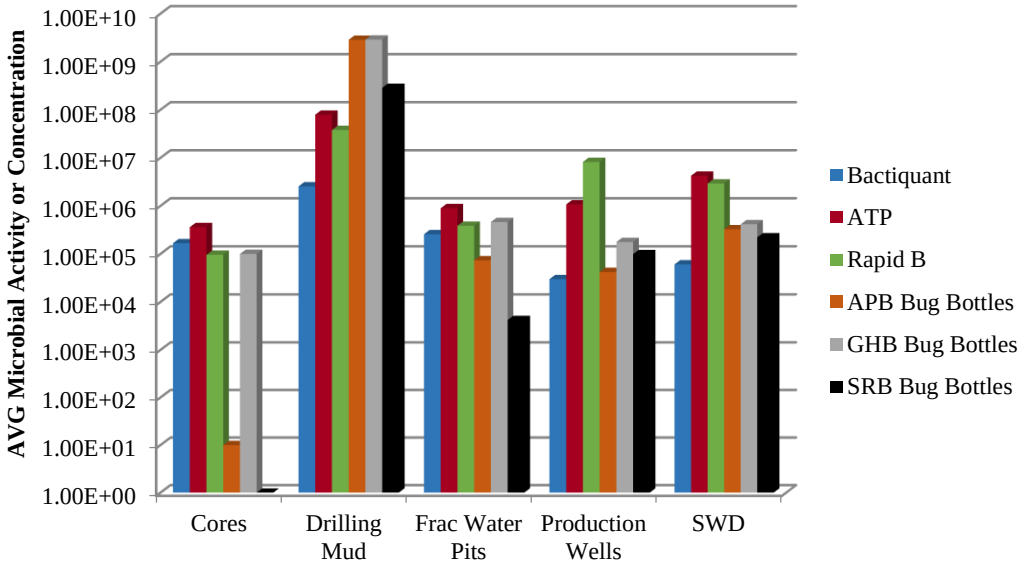

Figure 6. Shows a complete profile of the average microbial activity in Encana Oil & Gas (USA) Inc.'s production assets. Since the purpose of this study was to compare microbial values from the four different technologies, microbial concentrations were assumed to mean overall microbial activity or concentration and the individual units neglected. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

Considering the data from the Encana Oil & Gas (USA) Inc.'s Microbial Study, non-treated water is considered higher risk for microbial related corrosion and souring in production operations. Treatment of some form is understood from this study as a necessary component in future production operations. Low operating costs, unit mobility, and ease of use are key to any treatment's success when considering addition

to oil and gas operations. Additional microbial population information from production well, drilling mud, and fracturing water samples is listed in three metagenomic reports in (Appendix C). Additional microbial population information from filtration study is included as supplementary data to Encana Oil & Gas (USA) Inc. metagenomics reports in (Appendix F).

A&M Pilot Plant Testing with Pioneer Natural Resources

In addition to research provided by Encana Oil & Gas (USA) Inc. Corporation, membrane filtration proof of concept data was shared by Pioneer Natural Resources. Texas A&M researchers from the GPRI group were invited to demonstrate membrane treatment of produced water during production operations. Researchers used an MF membrane from Membrane Specialists, a Dow-spiral wound NF membrane, and a Nano Stone ceramic MF membrane to carry out filtration. Membranes were fitted inside a mobile filtration trailer and driven to the production site in Midland, TX. Table 4 listed below demonstrates microbial activity observed in both the feed and permeate waters during filtration treatment.

Table 4. Microbial Activity during Membrane Filtration

Sample	Method of Analysis	Date/Time of Collection	SPB (CFU/ml)	APB (CFU/ml)	GHB (CFU/ml)	IRB (CFU/ml)	SRB (CFU/ml)
Raw Feed	Commercial Lab	9/30/14 9:10 AM	66500	1500	<7000	1400	1200
MF Membrane Specialists Permeate	Commercial Lab	9/30/14 3:20 PM	<500	<100	<7000	<25	<200
NF Dow Spiral Wound Permeate	Commercial Lab	10/1/14 2:00 PM	<500	<100	<7000	<25	<200
Feed	Commercial Lab	10/2/14 11:50 AM	500	9000	<7000	263	700
Raw Feed	QuickChek SRB Assay	10/6/2014 9:35 AM					10^5
Raw Feed	QuickChek SRB Assay	10/7/14 9:50 AM					10^5
Raw Feed	QuickChek SRB Assay	10/7/14 10:36 AM					10^4
MF Nano Stone Permeate	QuickChek SRB Assay	10/7/14 11:06 AM					<10^3
MF Nano Stone Permeate	QuickChek SRB Assay	10/7/14 11:33 AM					<10^3
MF Nano Stone Feed	QuickChek SRB Assay	10/7/14 12:00 PM					10^4
MF Nano Stone Permeate	Commercial Lab	10/7/14 12:15 PM	<500	<100	<7000	<25	<200
MF Nano Stone Permeate	QuickChek SRB Assay	10/7/14 2:04 PM					<10^3
MF Nano Stone Feed	QuickChek SRB Assay	10/7/14 2:32 PM					10^5

Table 4 Continued

Sample	Method of Analysis	Date/Time of Collection	SPB (CFU/ml)	APB (CFU/ml)	GHB (CFU/ml)	IRB (CFU/ml)	SRB (CFU/ml)
MF Nano Stone Feed	QuickChek SRB Assay	10/7/14 4:12 PM					10^3
MF Nano Stone Permeate	QuickChek SRB Assay	10/7/14 4:32 PM					<10^3
Filtration Concentrate 25%	QuickChek SRB Assay	10/7/14 5:00 PM					10^5
MF Nano Stone Feed	Commercial Lab	10/8/14 9:15 AM	500	500	<7000	263	<200
MF Nano Stone Permeate	Commercial Lab	10/8/14 9:50 AM	<500	<100	<7000	2300	<200
MF Nano Stone Feed	Commercial Lab	10/8/14 10:05 AM	6500	1500	50000	263	<200

Microbial activity before and after membrane filtration. Water samples were collected throughout the nine hour filtration run over the course of five days. Commercial laboratory analysis results were reported as most probable number (MPN) and normalized using volume for (CFU/ml) units. SRB analysis was carried out using the QuickChek SRB assay produced by Modern Water. Values were verified by Commercial laboratory analysis.

According to Table 4, MF and NF membrane filtration appears to reduce microbial activity in produced water. This verifies that even the lowest level of membrane filtration is capable of reducing suspended microbial solids in produced waters. When evaluating SRB concentrations, the QuickChek assay appears to quantify SRB cells that are unable to grow on traditional culture media. This observation verifies the point addressed earlier in the literature review section. Traditional culture methods

have been documented to under estimate microbial populations due to the inability to culture all cells in a sample several days after collection. For this reason, accurate real-time microbial analysis kits should be used for quicker in field results. HACH BART bottles used to quantify SRB, SPB, GHB, APB, and IRB microbial populations during the Pioneer Natural Resources study also supports the need for real-time microbial analysis. Results from this test required nine days for complete analysis to be achieved. Quicker results would ensure more accurate microbial quantification and response to prevent well corrosion and souring.

Total dissolved (TDS) and suspended solids (TSS) values provided in Figure 7 also provide quantitative support for the enhancement of produced water quality during membrane filtration.

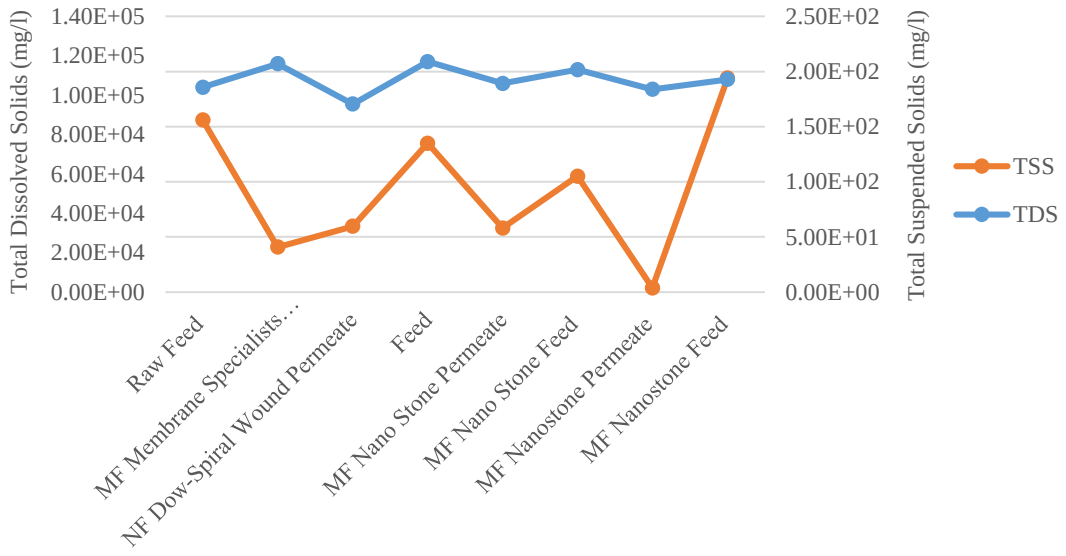

Figure 7. Shows total suspended and dissolved solids removal during membrane filtration treatment. Water samples were collected throughout nine hour filtration runs over the course of five days. Filtration units were operated in concentrate mode over the course of the five days. The higher TSS value observed for the NF Dow-Spiral Wound Permeate is due to running in concentrate mode for three hours after an overnight shutdown prior to sample collection. The higher TDS value observed for the MF Membrane Specialists Permeate is due to running in concentrate mode for five hours prior to sample collection. Consistent sampling was carried out once daily for the remaining samples.

Both the Encana Oil & Gas (USA) Inc. and Pioneer Natural Resources studies indicate that monitoring nitrogen in the form of ammonia, nitrate, and nitrite, organic carbon, and sulfate levels is common in oil field water analysis. The Encana Oil & Gas (USA) Inc. study directly associates these chemical components with microbial growth. This association further supports the need to determine if MF and NF membrane filtration is capable of reducing nutrient components as well as microbial activity.

CHAPTER III

METHODOLOGY

Research design, testing parameter selection, instrumentation, validity and reliability, collection of data, and quantitative analysis of analytical data will all be discussed within this section. For the purpose of this study, water stability is controlled microbial growth, this is indicated by reduced concentrations of microbial nutrients, improved dissolved oxygen concentration, and reduced microbial activity. Also, treated produced water is defined as membrane filtration treated water with no biocide added to chemically control microbial populations.

Research Design

The research design used for this study was both pilot scale and laboratory based with benchtop filtration and chemical analysis. Study parameters were selected to examine the efficiency of membrane filtration in producing stable reusable hydraulic fracturing water. The foundation of membrane treatment was based on the framework already established by the TEES Global Petroleum Institute (GPRI).

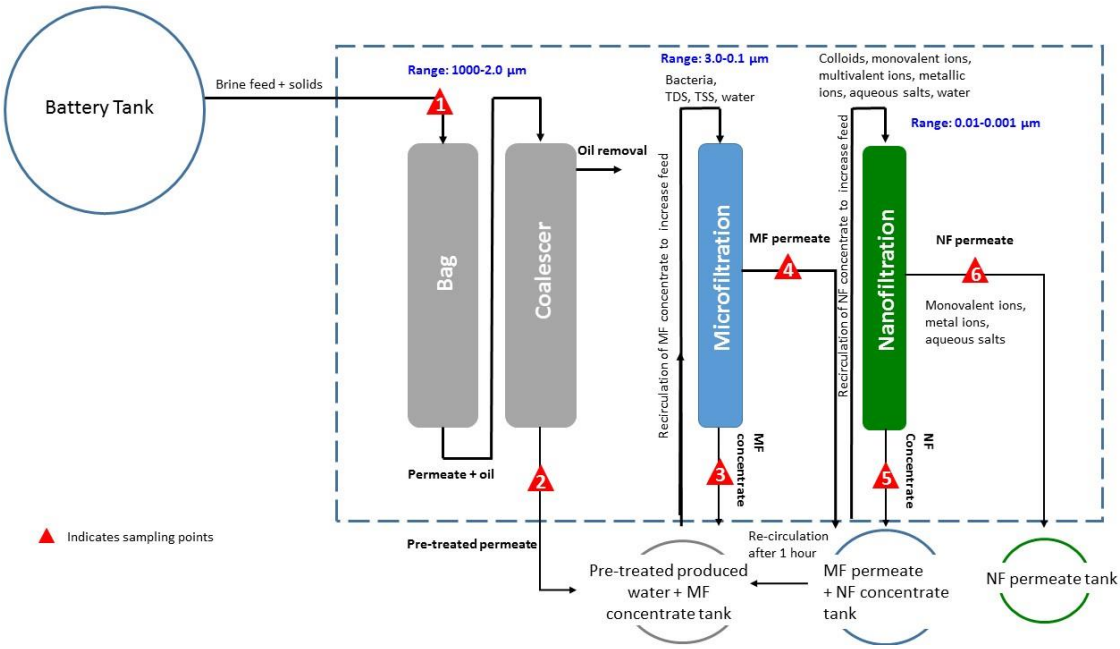

Figure 8. Produced water pretreatment, microfiltration, and nanofiltration filtration scheme.

Equipment and prior field knowledge was built on by the inclusion of bacterial monitoring during and after filtration treatment. Identification of microbial nutrient components within the water analysis scheme was also added to prior knowledge.

Operation Strategy and Sampling Locations

MF and NF systems were run in concentrate mode during each experiment for seven hours. The main objective of running in concentrate mode was to optimize the clean water recovery and minimize the volume of water going to disposal. Water quality sampling was carried out at points before and after each treatment module to determine removal efficiency. Figure 8 above marks the location and sequence samples were collected in during each experiment. Samples 1 and 2 mark the beginning of the sampling triage and were collected once per experiment. Samples 3-6 were collected

each time 5 gallons of permeate was collected and transferred to the NF system. During each filtration run, sample collection was adjusted according to permeate flow rate resulting from the different produced water qualities. Samples for equipment failure run: 1 raw feed, 1 pretreat, 4 MF permeates, 3 concentrates, 1 NF raw feed (due to more than 24 hours since MF run), 1 NF permeate, and 1 NF concentrate. Samples for trial one run: 1 raw feed, 1 pretreat, 4 MF permeates, 2 concentrates, 2 NF permeates, and 2 NF concentrates. Samples for trial two run: 1 raw feed, 1 pretreat, 3 MF permeates, 3 MF concentrates, 3 NF permeates, and 3 NF concentrates.

Once samples were collected, analysis was carried out within 2-3 hours to ensure real-time monitoring of water stability. When storage was necessary, samples were sealed and placed in a 4˚C refrigerator for no more than 6 hours before analysis was completed. Samples collected for commercial laboratory analysis were submitted for testing within three hours of collection. Analysis results were received within one week of the date of sample submission. All required waste disposal procedures for hazardous wastes were carried out to ensure compliance with TAMU Health and Safety.

Testing Parameter Selection

The parameters selected for sample analysis during the study were determined from field data collected from studies carried out with Encana Oil & Gas (USA) Inc. Corporation and Pioneer Natural Resources in Midland, TX. The Encana Oil & Gas (USA) Inc. study focused primarily on microbial activity in raw untreated produced waters, drilling muds, and formation cores. Data from this study shows the breadth and

severity of microbial activity in oil and gas operations. Upon completing analysis of this study, it was determined that Mycometer's Bactiquant meter was the best real time analysis technology available for metabolic monitoring of microbial activity.

The Pioneer Natural Resources study was primarily focused on determining the efficiency of MF and NF treatment of produced waters. Data from this study demonstrates the solids removal potential of MF and NF treatment. Combined, both studies demonstrated a need for microbial monitoring and nutrient reduction through microfiltration and an added step of nanofiltration.

Instrumentation

Experimental Filtration Unit Setup

Membrane treatment experiments were conducted in a warehouse facility using both an MF and NF cross-flow filtration unit connected in sequence. Running both units in sequence simultaneously allowed enough time for each system to reach pseudo steady-state prior to sampling permeate and concentrate waters. Produced water was collected from a local SWD and stored in a 60 gallon polyethylene tank during the duration of each filtration run. Each batch of water collected can be considered chemically unique to each experiment. Experiments were run for seven hours without stopping to simulate a full scale treatment operation and to minimize microbial activity in permeate water post filtration.

Pretreatment was carried out using a Pentair Industrial 10 µm bag filter, model BP_420_10, followed by a Polymer Ventures hydrocarbon removal cartridge 2.75"x

20”. MF filtration was carried out using a 0.2 μm Corning ceramic membrane. NF was carried out using an Ultura nanofiltration membrane, model number NF2. The NF membrane was chosen because it still exhibited 98.0 % rejection of $MgSO_4$ and 55.0 % rejection of NaCl with a water flux of 64 (gallons/ft^2/day)/psi g (GFD). This was the only NF membrane Ultura sold with a high rejection rate and water flux greater than 60 GFD. The water flux implies the NF membrane requires a lower operating pressure and less energy for treatment. Figure 8 below displays the filtration scheme used during each filtration run, and outlines the system components under study. Photos showing both the MF and NF filtration units are included in (Appendix A).

Detailed information about the membranes used in each phase of the study is provided in Table 5 listed below.

Table 5. Filtration Unit Specifications for Trials 1-3

Membrane Specification	Comments
MF Membrane	0.2 μm Corning Ceramic
Trial 1 NF Membrane	GE Infrastructure Water & Process Technologies SEPA CF TF (Thin Film) NF; Membrane Type: HL Part Number: 1221923
Trial 2-3 NF Membrane	Ultura NF polymer membrane 98.0% rejection of MgSO4; 55.0% rejection NaCl; 64 gal/d/ft^2 (109 L/hr/m^2) minimum water flux; max temperature 104ºF; max pressure 600 psi; pH 10.5-2
MF Membrane Surface Area	Not Available
NF Membrane Surface Area	Contacting company
MF Power	110 volt single phase
NF Power	110 volt single phase
MF Feed Back Pressure	20 psi

Table 5. Continued

Membrane Specification	Comments
NF Feed Back Pressure	(Failure) 160, (Trials 1-2) 200 psi
MF Feed Flow Rate	8 gallons per minute (gpm)
NF Feed Flow Rate	1 gallons per minute (gpm)
MF Permeate Flow Rate	Graduated cylinder and stop watch
NF Permeate Flow Rate	Graduated cylinder and stop watch
MF Temperature	Thermocouple/ Thermometer
NF Temperature	Thermocouple
Number of MF Modules Per Trial	1
Number of NF Modules Per Trial	1
Feed Container	60 gallons; continuously mixed by flow
Pretreat/Concentrate Container Size	60 gallons; non-mixed

Specifications for membranes used during failure test, trail 1, and trial 2.

Failure Test Run Procedures

During the first experiment, the feed water flow rate for the MF treatment system was maintained at 8 gallons per minute (gpm). MF permeate flow rate was measured by hand and determined to be 0.10 gpm. Hand measurements were carried out using a graduated cylinder and stop watch. Samples were collected for 1 minute and converted to gpm for reporting purposes. MF feed pressure, the pressure required to push the concentrated feed water through the membrane, was adjusted to 20 psi. Upon completing MF filtration, the water was then fed directly into the NF system where the feed pressure was adjusted to reach 160 psi. NF feed pressure was gradually increased from a starting pressure of 120 psi to 160 psi over the course of 4 hours. The resulting feed flow rate achieved once the NF system reached pseudo pseudo steady state (determined to be at 160 psi) was 0.99 gpm. Constant pressure of 160 psi was chosen because, it was

determined to be the optimum pressure yielding the highest permeate flux with an acceptable rate of fouling. The resulting NF permeate flow rate was measured by hand and determined to be 0.002 gpm (high probability of inaccuracy at this range). Slow adjustments to the NF feed pressure have been identified in previous studies as key to avoiding fouling that could significantly reduce the operational life of the membrane.[20] Both feed and permeate flow rates were maintained throughout the course of the filtration run to keep the treatment process at pseudo steady state. Both the NF and MF feed streams were run in concentrate mode to enhance treated water yield.

In order to simulate a minor malfunction or "failure" water treatment was started during the morning hours around 9:00AM, and stopped for approximately 1 hour. Treatment was continued after the 1 hour downtime and stopped at 5 PM. Microfiltration permeate water was collected, sealed, and stored in a cold room 4ºC for 4 days. Cold storage was used in an effort to slow bacterial growth to a level that would still allow accurate quantification after storage. The 4 day downtime was intended to simulate a major equipment failure. Microfiltration permeate water was removed from the cold room and run through the nanofiltration system.

Filtration Trial 1 Run Procedures

During the second experiment, the feed water flow rate for the MF treatment system was maintained at 8 gallons per minute (gpm). MF permeate flow rate was measured by hand and determined to be 0.68 gpm. Hand measurements were carried out using a graduated cylinder and stop watch. Samples were collected for 1 minute and

converted to gpm for reporting purposes. MF feed pressure, the pressure required to push the concentrated feed water through the membrane, was adjusted to 20 psi. Upon completing MF filtration, the water was then fed directly into the NF system where the feed pressure was adjusted to reach 200 psi. NF feed pressure was gradually increased from a starting pressure of 50 psi to 200 psi over the course of 1 hour. The resulting feed flow rate achieved once the NF system reached pseudo steady state (determined to be at 200 psi) was 1 gpm. Pseudo steady state pressure of 200 psi was chosen because, it was determined to be the optimum pressure yielding the highest permeate flux with an acceptable rate of fouling. The resulting NF permeate flow rate was measured by hand and determined to be 0.02 gpm. Both feed and permeate flow rates were maintained throughout the course of the filtration run to keep the treatment process at pseudo steady state. Both the NF and MF feed streams were run in concentrate mode to enhance treated water yield.

Filtration Trial 2 Run Procedures

During the third experiment, the feed water flow rate for the MF treatment system was again maintained at 8 gpm. MF permeate flow rate was measured by hand and determined to be 0.37 gpm. MF feed pressure was adjusted to 20 psi. Upon completing MF filtration, the water was fed directly into the NF system and the feed pressure was adjusted to reach 200 psi. NF feed pressure was gradually increased over the course of 1 hour. The resulting feed flow rate achieved once the NF system reach pseudo steady state (determined to be at 200 psi) was 1.12 gpm. The resulting NF permeate flow rate

was measured by hand and determined to be 0.06 gpm. Both feed and permeate flow rates were maintained throughout the course of the filtration run to maintain a pseudo steady state system. Both the NF and MF feed streams were run in concentrate mode to enhance treated water yield.

Chemical Analysis Instrumentation

Sample analysis involved the use of the following chemical instruments:

- HACH HQ40d meter and PH10105 probe – pH analysis.
- HACH HQ40d meter, HACH HR IntelliCAL ISECL181, and HACH Chloride ionic strength adjustor (ISA) Buffer Powder Pillows – Chloride analysis
- HACH 2100P Turbidometer – Turbidity analysis.
- HACH Spectrophotometer DR 5000, HACH Potassium method 8049, and required chemicals – potassium analysis.
- HACH spectrophotometer DR 5000, HACH sulfide method 8131, and required chemicals – sulfide analysis.
- HACH spectrophotometer DR 5000, HACH sulfate method 8051, and required chemicals – sulfate analysis.
- HACH spectrophotometer DR 5000, HACH total iron (ferrous iron without digestion) method 8008, and required chemicals – total dissolved iron analysis.
- HACH spectrophotometer DR 5000, HACH DRB200 reactor, HACH N (HR) method 10072, and required chemicals – total nitrogen analysis.
- HACH titration kit for method 10253, and required chemicals – calcium analysis.

- HACH titration kit for method 10247, and required chemicals – total hardness analysis.
- HACH spectrophotometer DR 5000, HACH DRB200 Reactor, and required chemicals for method 8190 – total phosphorus analysis.
- Bactiquant-water Meter, 5 pack test kit containing filters, substrate, blunt tipped needles, standard, neutralizer, and cuvettes – microbial activity analysis.
- Fischer Scientific Accumet AP74 DO meter – dissolved oxygen analysis.
- GE InnoVox TOC analyzer – total carbon (TC), total inorganic carbon (TIC), and total organic carbon (TOC) analysis.

(Table 6) below illustrates the chemical analysis carried out during the failure test run.

Table 6. Failure Test Chemical Analysis

Total Nitrogen (TNT)	Total Phosphate	TC/IC/OC
Total Hardness	Total Iron	Sulfate
Sulfide	Potassium	Magnesium
Bactiquant	pH	Chloride
Dissolved Oxygen	Turbidity	Temperature
Calcium		

Failure test chemical analysis was used to determine which tests were more feasible to run with a one person analysis team. pH, turbidity, temperature, chloride, dissolved oxygen, and microbial activity was monitored at pilot plant location. TC/IC/OC, sulfate, magnesium, potassium, total iron, total phosphate, total hardness, total nitrogen, sulfide, and calcium were monitored at graduate laboratory.

HACH chemical analysis was carried out for comparison purposes along with commercial laboratory analysis by the Texas A&M AgriLife Extension Soil, Water and

Forage Testing Laboratory. Table 7 below illustrates the chemical analysis carried out during trials 1 and 2.

Table 7. Trial 1 and 2 Chemical Analysis

Total Nitrogen (TNT)	Sulfide	TC/TIC/TOC
Bactiquant	pH	Chloride
Dissolved Oxygen	Turbidity	Temperature

Trial 1 and 2 chemical analysis was determined to be feasible for a one person analysis team when started directly after sample collection. pH, turbidity, temperature, chloride, dissolved oxygen, and microbial activity was monitored at pilot plant location. TC/TIC/TOC, sulfate, total nitrogen, and sulfide were monitored at graduate laboratory.

HACH chemical analysis discontinued for trial 1 and 2 was carried out by commercial laboratory, the Texas A&M AgriLife Extension Soil, Water and Forage Testing Laboratory.

Data Validity and Reliability

Data validation and reliability was carried out by using commercial laboratory results to verify HACH data. Discrepancies between values resulted in the decision to report only commercial laboratory data for all chemical analysis discontinued in trials 1 and 2. The remaining chemical values tested for during trials 1 and 2 were compared to commercial laboratory results without any discrepancies. All chemical testing procedures were followed to exact specifications to ensure accurate results. All chemical testing kits were ordered one month prior to testing to ensure fresh chemicals for all testing.

Bactiquant analysis was carried out under the direction of Lisa Rogers, the President of Mycometer. Testing procedures were developed under her direction to ensure quality repeatable results.

Quantitative Analysis of Data

Data was compiled electronically according to filtration trial. Concentration units were compared and converted if not uniform to mg/l. Each trial's data was used to construct graphs to better visualize changes before, during and after filtration treatment. Consolidation of data into uniform graphs containing all three trials resulted in the averaging of replicates for a total of three points per chemical component measured per trial: raw feed, pretreat, permeate, and concentrate.

Microbial activity was normalized by the volume used during testing to convert the BQV into a concentration value BQV/ml. Volumes used for normalization are the following: failure test normalization value – 60ml, trial 1 normalization value – 10ml (for raw feed, pretreat, and MF samples) 50ml (for NF samples), trial 2 normalization value - 10ml (for raw feed, pretreat, and MF samples) 50ml (for NF samples).

Open air and sealed MF treated produced water microbial growth tests were graphed to determine effect of oxygen on microbial activity of treated water. MF and NF 8 hour growth tests were carried out to determine if treated waters exhibited the classic microbial growth phases when exposed to environmental microbes during storage.

Select chemical components exhibiting a significant change post membrane treatment were graphed for each trial. Chemical components selected and graphed are

the following: inorganic carbon, total organic carbon, total elemental sulfur, sulfate, nitrate, total hardness, magnesium, calcium, bicarbonate, total elemental phosphorus, total soluble iron, manganese, dissolved oxygen, microbial biomass (activity), and conductivity. Inorganic carbon showed some deviation when compared to commercial laboratory bicarbonate values. Due to this inorganic and organic carbon were divided by their respective raw feed values and multiplied by one hundred to determine percent removal. Graphs of percent removal for each were constructed to use TIC and TOC data without contradicting commercial laboratory results.

Completing graphical analysis, two graphs were constructed to display the effects of MF and NF membrane treatment on potential electron donor and potential electron acceptor species present in produced waters from all three trials. The following chemical components were considered potential electron donors: total soluble iron, ammonium, ammonia, and nitrite. The following chemical components were considered potential electron acceptors: manganese, nitrate, dissolved oxygen, and sulfate.

In an effort to better identify MF and NF treated water stability for storage, nutrient ratios were calculated. Carbon was used as the base nutrient and all additional nutrient components were divided against carbon and multiplied by one hundred to calculate a percent composition with respect to carbon. Nitrogen, sulfur, phosphorus, sulfate, iron, manganese, and dissolved oxygen were used to calculate nutrient ratios for treated produced waters.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter includes a presentation and discussion of the findings of this study by objective.

a. Microbial water stability during suspended treatment
    i. Equipment failure
    ii. Storage in open and sealed containment
b. Water stability when treating continuously
    i. Microbial activity post continuous treatment
    ii. Reduction of microbial electron donors and acceptors
    iii. Reduction of dissolved organic and inorganic carbon
    iv. Total hardness reduction
    v. Microbial nutrient levels post filtration treatment with MF and NF systems

Findings Related to Objective One: Water Stability When Treating Continuously

Objective One is meant to describe the effects of continuous treatment on the biological and chemical water quality parameters of the produced water.

Microbial Activity Post Continuous Treatment

Optimum removal of bacteria, and divalent ions is expected when running both MF and NF technologies in a continuous process. Real-time analysis allows microbial monitoring as the water passes through each filtration unit during operation. Figure 9 shows the microbial activity in permeate waters collected from trials 1 and 2 as compared to the failure test.

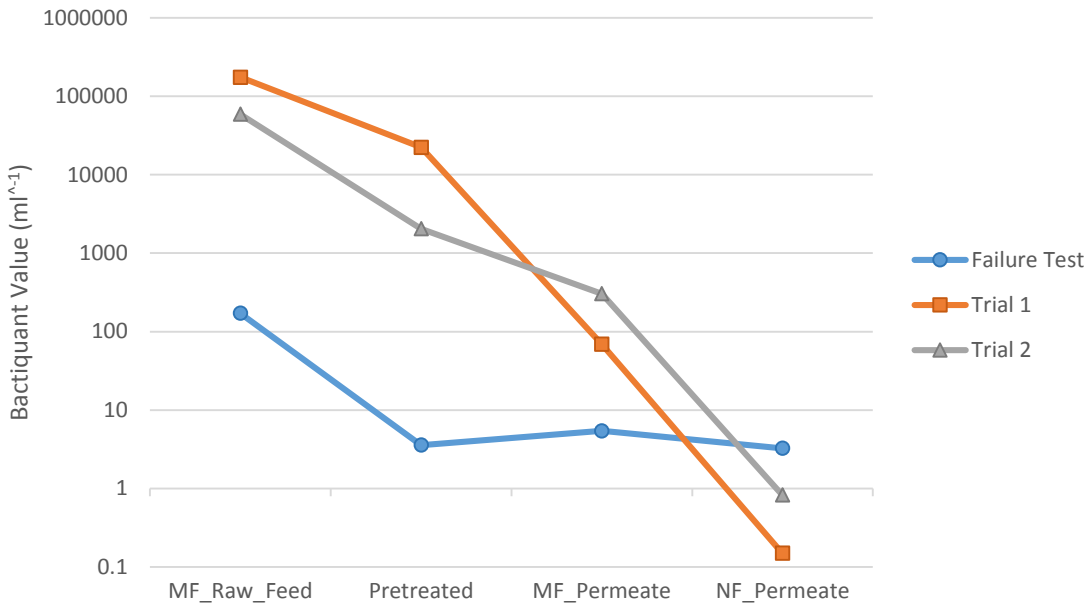

Figure 9. Microbial activity in permeate waters from MF and NF continuous treatment. Microbial activity from the failure test was included to compare microbial levels from continuous treatment to levels from start and stop treatment "failure test".

Microbial activity is observed to reduce as filtration progresses. Compared to the failure test results shown in Figure 9 above, continuous running shows better efficiency in reducing microbial activity in treated produced waters. This supports the expected results listed prior to experimental analysis for MF solids removal. The graphical analysis shown in Figure 9 also emphasizes the need to monitor bacterial levels during

the treatment process to ensure high quality permeate waters. Quality control after all is also important in oil and gas field operations.

Reduction of Microbial Electron Donors and Electron Acceptors

Although reduction of microbial activity is important, reduction of microbial electron donors and acceptors is also an important category to monitor. High levels of electron donors and acceptors in the permeate waters post treatment means that treated waters can still support high levels of microbial activity after treatment. Reduced populations will therefore be expected to re-grow. Treated waters can also support microbial populations from the environment that find their way into treated waters during storage or transport. Because of this, the following which were considered microbial electron donors were monitored: total soluble iron, ammonium, ammonia, total organic carbon based compounds and nitrite. Total nitrogen was used to determine the nitrogen electron donors. Total soluble iron was considered to be an electron donor. Although total soluble iron does not differentiate between ferric and ferrous iron, ferrous iron will likely be the dominant soluble iron species. Ferric iron is largely insoluble when not complexed with other water soluble components. The following were considered important microbial electron acceptors: manganese, nitrate, dissolved oxygen, and sulfate. Figure 10 and 11 listed below illustrate the findings for removal of electron donors and acceptors in trials 1 and 2.

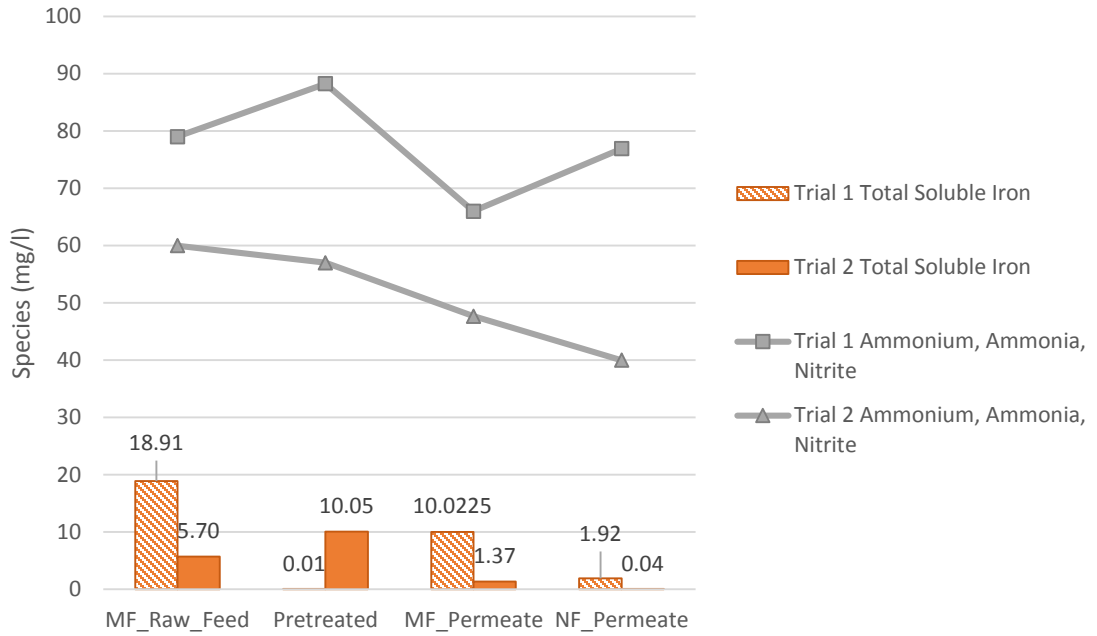

Figure 10. Potential electron donors present after MF and NF treatment of produced waters. Reduction graphed with respect to system running in concentrate mode. Values for ammonium, ammonia and nitrite were obtained by subtracting nitrate values (obtained by commercial laboratory testing) from the total nitrogen values.

Overall reduction of electron donor concentrations were observed concluding MF and NF treatment of produced water from both trials 1 and 2. Ammonium, ammonia, and nitrite cycling can be directly attributed to microbial activity prior to MF treatment and NF treatment as displayed by the graph in Figure 10. MF and NF pore sizes do not allow the removal of monovalent ions such as ammonium, ammonia, and nitrite. However, reduction of soluble iron can be directly attributed to NF treatment during water processing. NF pore sizes allow the removal of divalent and polyvalent ions such as iron (II) and iron (III). Although both trials were run the same, the produced water composition for each trial was remarkably different.

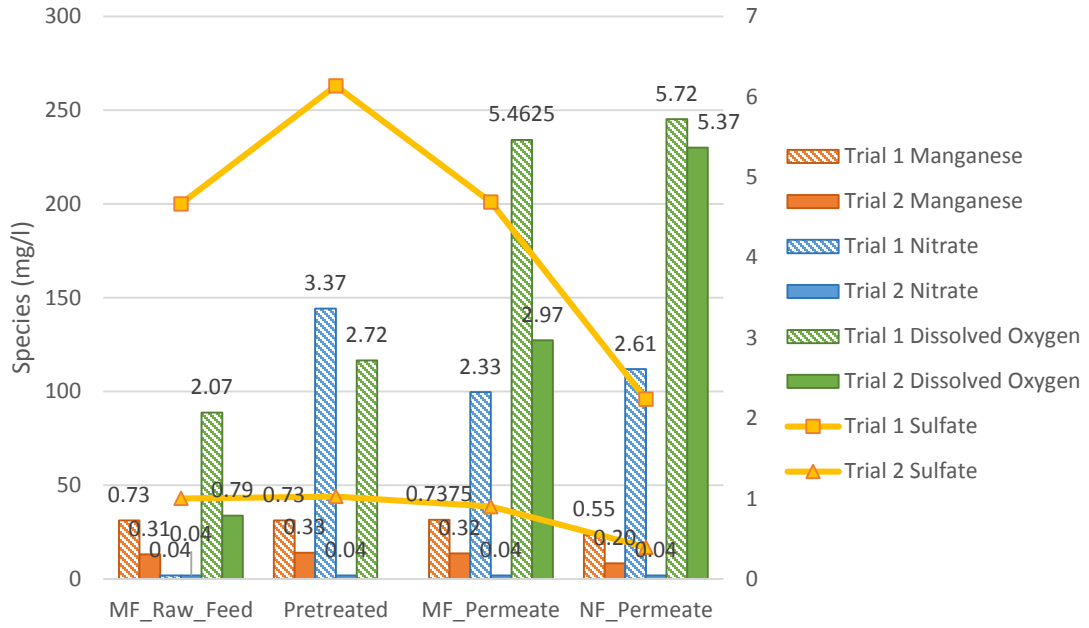

Figure 11. Potential electron acceptors present after MF and NF treatment of produced waters. Reduction graphed with respect to system running in concentrate mode.

The data displayed in Figure 11 shows the removal of manganese, and sulfate after treatment with NF. NF pore sizes were expected to select for divalent and polyvalent ions as described for Figure 10. Nitrate also appears to cycle as a result of both redox chemistry and microbial activity during both MF and NF treatment during both trials 1 and 2. Dissolved oxygen increases progressively as water treatment progresses. Increasing dissolved oxygen concentrations support enhanced water quality as a result of filtration treatment.

Both microbial electron acceptors and donors appear to be lower as a result of microbial activity prior to MF treatment and treatment with the NF technology. Therefore, it can be expected that water stored in a tank with little fluid motion will have more change in dissolved oxygen and nitrate concentrations than water passing through

a membrane. Although MF treatment appears to only reduce solids and some microbial metabolites, MF treatment is essential to increase NF treatment efficiency for the removal of the remaining microbial metabolites. Well established pretreatment downstream of higher level filtration stages will ensure better treated water quality and lower equipment costs over the operational lifespan. Treated water resulting from both trial 1 and 2 appear to exhibit better storage capability as a result of lower microbial activity and microbial metabolite concentrations than raw untreated produced waters. Additional graphs are provided for a better understanding of microbial nutrients measured in all three trials in (Appendix E).

Reduction of Dissolved Organic and Inorganic Carbon

Carbon in the form of organic and inorganic species can also attribute to microbial growth. Although this process is species dependent, aerobic and anaerobic metabolic activity can utilize carbon as a driving force. Dissolved carbon species can serve as both a carbon source, an electron donor and an electron acceptor during anaerobic fermentation.[29] During aerobic metabolism, dissolved carbon species commonly serve as carbon sources and electron donor.[29] Since dissolved carbon can play multiple roles in species dependent microbial metabolism and microbial activity was generalized, carbon was graphed as its own category. Figure 12 below displays the percent removal of total dissolved organic carbon quantified during trials 1 and 2.

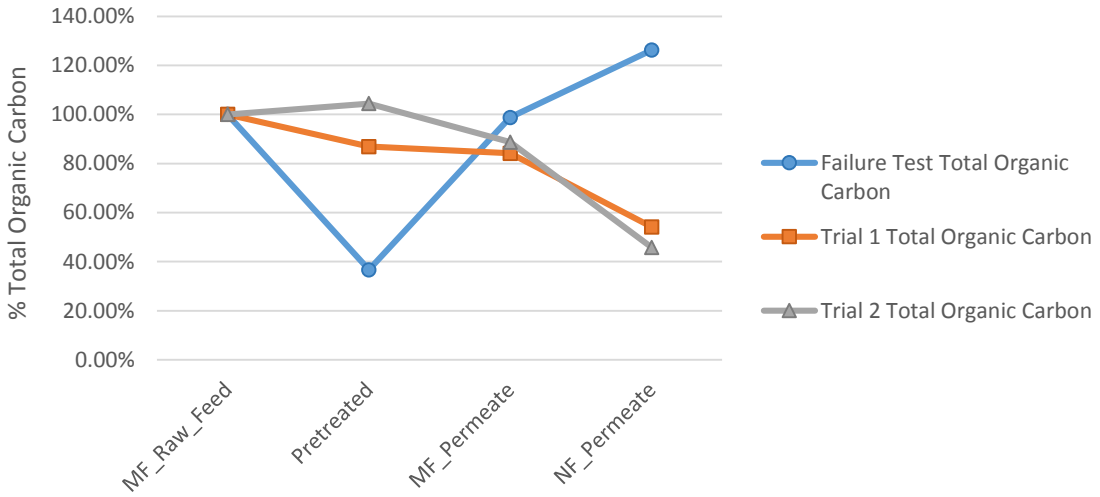

Figure 12. Percent removal of total organic carbon after MF and NF treatment. Reduction graphed with respect to system running in concentrate mode.

Continuous processing appears to more efficiently reduce dissolved organic carbon concentrations than the start stop treatment observed in the failure test. The increase in dissolved organic carbon between MF and NF treatment is likely due to microbial activity. Metabolism of large carbon species still present in MF permeate during the downtime likely produced smaller linear carbon species. Enhanced microbial activity in failure test waters can be expected as a result of the increased dissolved organic carbon levels. Figure 13 below displays the percent removal of total dissolved inorganic carbon quantified during trials 1 and 2.

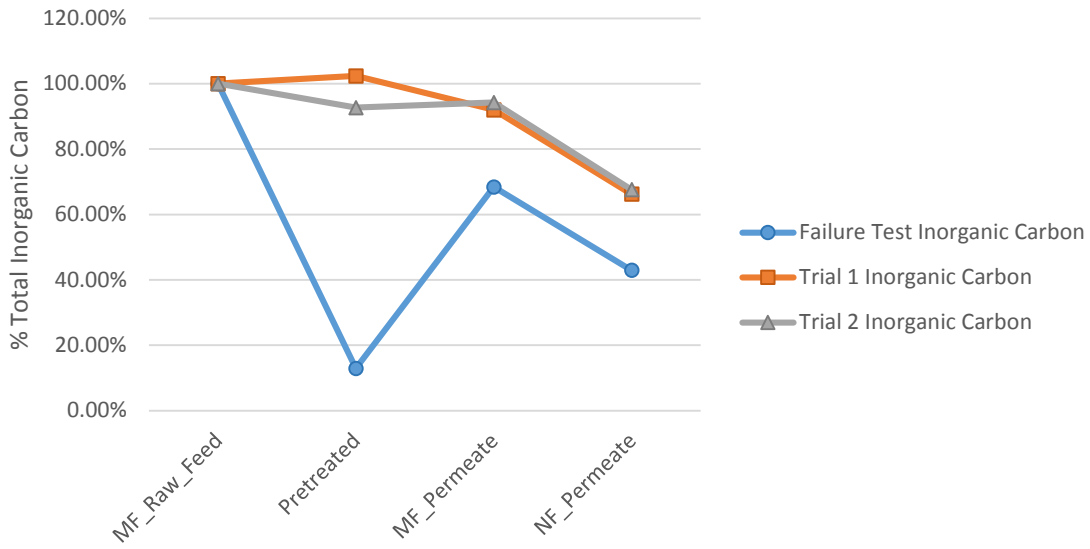

Figure 13. Percent removal of total inorganic carbon after MF and NF treatment. Reduction graphed with respect to system running in concentrate mode.

Dissolved inorganic carbon concentrations appear to decrease consistently as a result of filtration treatment. Dissolved inorganic carbon includes carbonic acid, carbonate, and bicarbonate species involved in water buffering. Since the pH of treated water increases as a result of treatment and increased dissolved oxygen levels. The decrease in inorganic carbon in the form of bicarbonate can be attributed to atmospheric carbon dioxide equilibration.[12] Although dissolved inorganic carbon can be used as a carbon source in microbial activity such as nitrification. Microbial activity is not the sole contributor to the decrease observed after MF treatment. Data listed in both Figures 12 and 13 again show a better water quality post treatment of raw produced waters with MF and NF filtration technologies.

Total Hardness Reduction

Though calcium and magnesium do play a role in microbial activity, both ions are known to play a more important role in water hardness.[27, 51-53] Scaling as a result of high levels of calcium and magnesium in produced waters is commonly observed in equipment used in water transport and processing. Due to this, reduction of these scaling ions is thought to enhance water quality for industrial use. Figure 14 displayed below shows the total hardness removal efficiency for the MF and NF filtration treatment system used in the study.

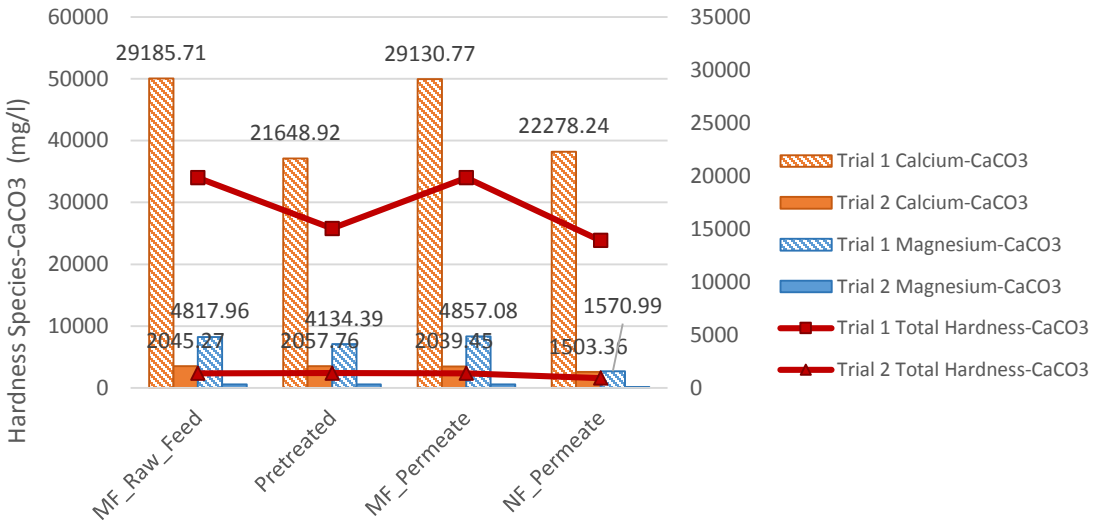

Figure 14. Removal of total hardness during filtration treatment trials 1 and 2. Reduction graphed with respect to system running in concentrate mode.

Lower levels of calcium and magnesium concentrations are observed after NF treatment as expected. NF pore size selects for the removal of divalent and polyvalent ions such as calcium and magnesium. Again as demonstrated before, MF treatment is best for solids removal and microbial activity reduction as a result microbial metabolite reduction. Reduction of total hardness is evident from total hardness data graphed for

trials 1 and 2. Data from these trials confirm NF treatment's ability to reduce calcium and magnesium ion concentrations. Water with reduced scaling potential is therefore observed as a result of NF treatment of raw produced waters. Additional graphs are provided for a better understanding of microbial nutrients measured in all three trials in (Appendix E).

Microbial Nutrient Levels Post Filtration Treatment with MF and NF Systems

Overall water stability of treated waters is best classified by total nutrient ratio tables. The total nutrient ratios were determined by comparing the mass concentration of the nutrient in question to the mass concentration of total carbon. Tables 8 and 9 best display the reduction of microbial nutrients post treatment for all three trials.

Table 8. **Nutrient Ratios for Microfiltration Permeate**

Nutrient	Failure Test	Trial 1	Trial 2
Carbon:	100.00%	100.00%	100.00%
Nitrogen:	NA	85.12%	16.25%
Sulfur:	17.12%	85.93%	4.40%
Phosphorus:	3.30%	14.54%	3.41%
Sulfate:	51.24%	257.28%	13.18%
Iron:	0.10%	12.83%	0.47%
Manganese:	0.45%	0.94%	0.11%
Oxygen:	NA	6.99%	1.01%

Nutrient percentages were calculated with respect to carbon for all three trials.

Table 9. Nutrient Ratios for Nanofiltration Permeate

Total Nutrient	Failure Test	Trial 1	Trial 2
Carbon:	100.00%	100.00%	100.00%
Nitrogen:	21.27%	146.36%	23.35%
Sulfur:	1.36%	60.56%	3.31%
Phosphorus:	1.61%	20.76%	3.48%
Sulfate:	4.07%	181.30%	9.92%
Iron:	0.02%	3.63%	0.02%
Manganese:	0.09%	1.04%	0.11%
Oxygen:	3.29%	10.80%	3.13%

Nutrient percentages were calculated with respect to carbon for all three trials.

Concluding treatment, it was observed that carbon, nitrogen, sulfur, phosphorus, and sulfate makeup the largest percentage of nutrients found in treated produced waters. Although treatment is successful in reducing nutrient levels, the overall activity potential for treated waters is still relatively high.

Results Related to Objective Two: Microbial Water Stability During Suspended Treatment and Storage

Objective Two is meant to describe the effects of storage on microbial activity under both aerobic, and anaerobic conditions during periods of suspended treatment activity “downtime”.

Equipment Failure

The purpose of analyzing the effects of downtime on biomass activity is to emphasize the non-stop microbial action that occurs during this period. Although treatment is suspended, water is still continuously reacting both chemically and biologically. Figure 15 displays microbial activity during both the minor and major equipment failure simulations.

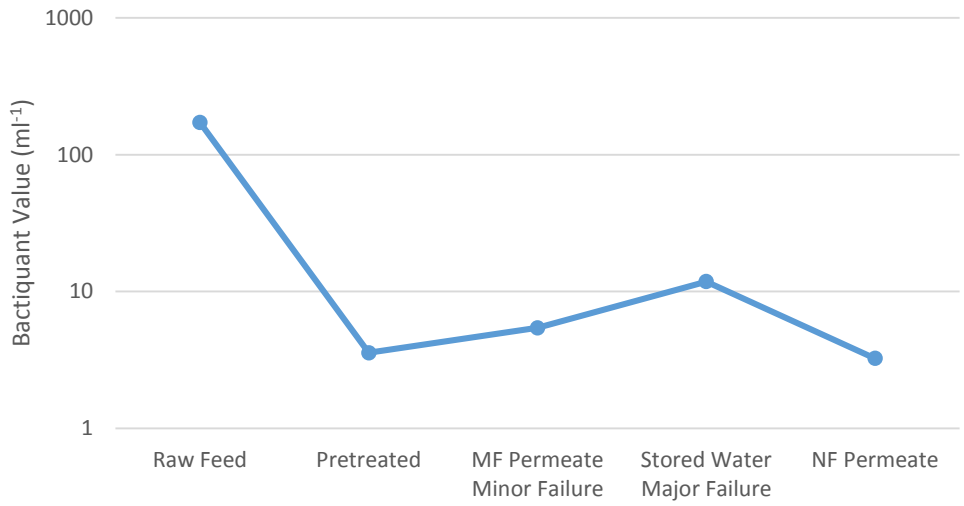

Figure 15. Microbial activity during both minor (1hour) and major (4 day) equipment failure simulations during the failure test. The minor failure simulation was carried out during MF treatment. The major failure simulation was carried out between the MF and NF treatments. The stored water value reflects major failure. Also note that pretreatment was carried out one day prior to filtration running, higher microbial values for pretreatment reflect this.

The results provided in Figure 15, show that periods of treatment suspension can allow microbial activity to become reestablished at higher levels than treated values at the start of storage. High nutrient levels in produced waters is thought to be the main contributor to microbial reestablishment.

Storage in Open and Sealed Containment

Storage analysis under aerobic and anaerobic conditions is meant to show microbial activity during transport and storage in well operations. Figure 16 displays the microbial activity during both aerobic and anaerobic conditions.

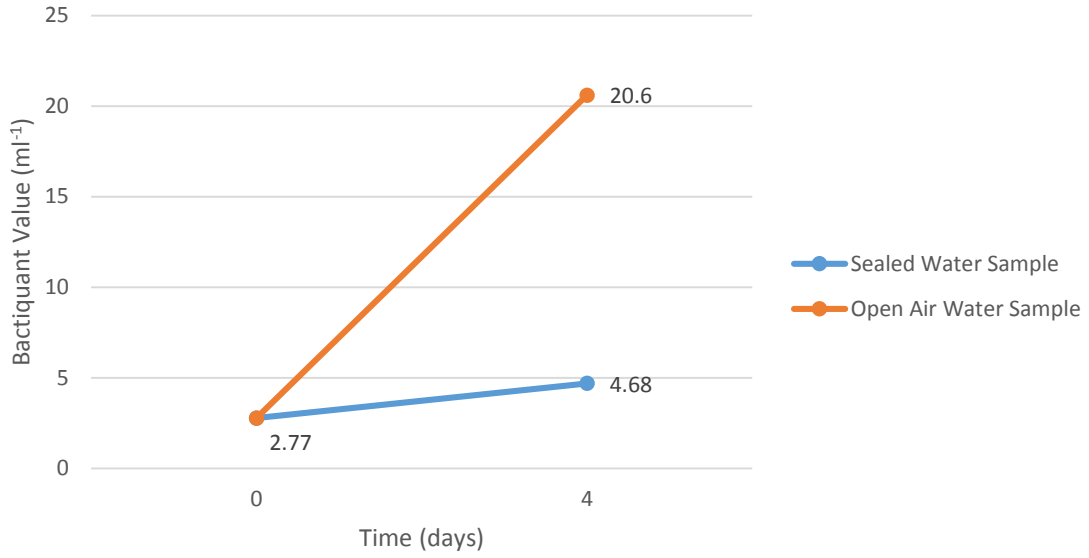

Figure 16. Aerobic and anaerobic simulation of storage conditions during transport and storage during field operations. Water used for both conditions was pretreated by MF filtration.

Since oxygen is a more favorable electron acceptor than organic compounds, rapid microbial growth is expected under aerobic conditions.[12, 29] Microbial activity as a result of fermentation is expected to occur at a slower rate. Graphical results agree with this published finding. Higher microbial activity is observed in the presence of oxygen, and lower activity in the absence of oxygen. Therefore, it is expected that open air fracking water storage pits will exhibit a much higher microbial activity than sealed containments. Figure 17 and 18 illustrate the percent change experienced by microbial

substrate as a result of chemical and biological activity during open air and sealed storage.

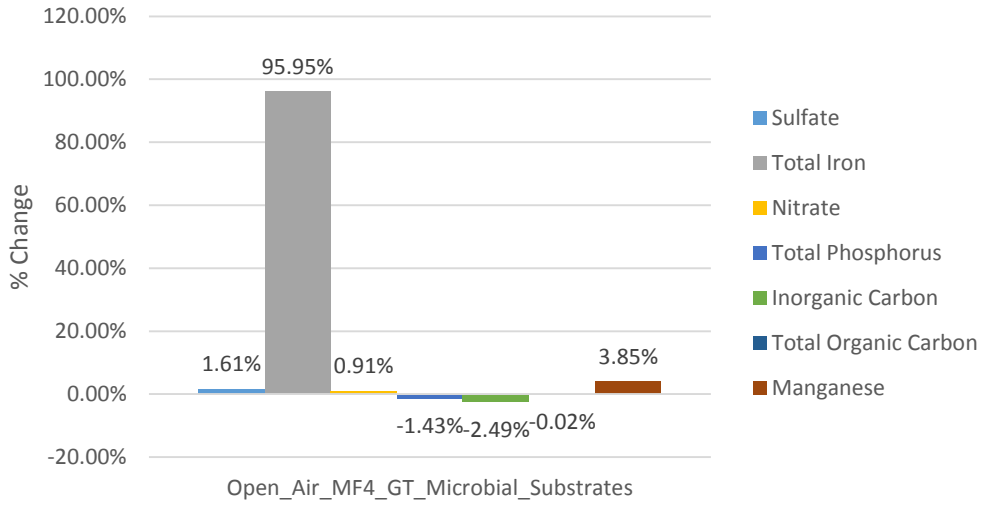

Figure 17. Percent change during four day open air storage growth test.

Redox reactions appear to play an active role for total iron during storage, but a less active role for most of the substrate listed in Figure 17. Positive change indicates the occurrence of reduction reactions which lead to an overall increase in substrate concentration levels during the growth test. Negative change indicates possible microbial influence causing an overall decrease in substrate concentration during the growth test.

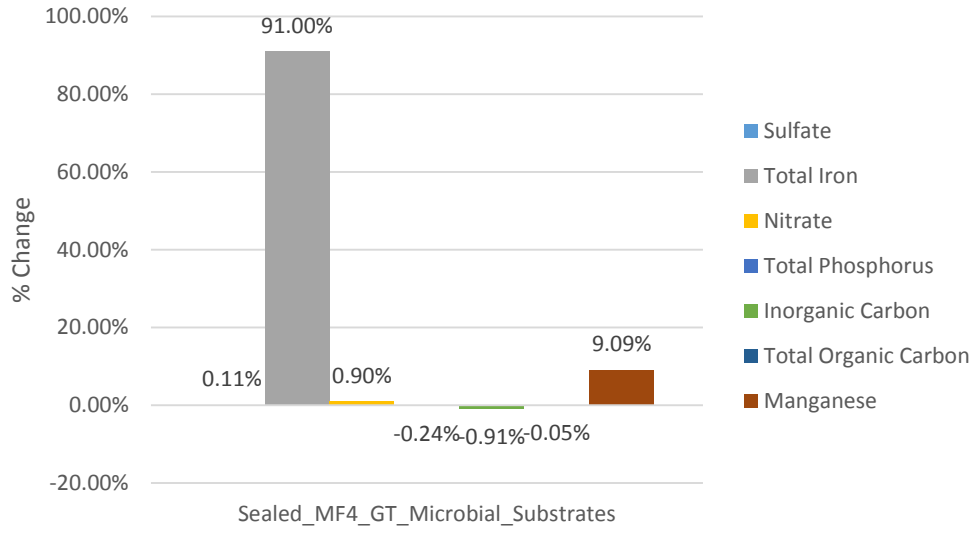

Figure 18. Percent change during four day sealed storage growth test.

Additional concentration data is included for each growth test in (Appendix D). Dissolved oxygen was not measured during this growth test because the dissolved oxygen probe was being shipped.

Additional information is also included for an eight hour growth test conducted during trial 2. Data is included in graphs in (Appendix D). Although the information provided in these graphs is informative, the data is not useful due to the generalized quantification of microbial activity. For future studies, species specific growth tests would be useful to quantify microbial populations to determine high, medium, and low risk population concentration values.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

This study analyzed the perceived microbial monitoring needs of the oil and gas industry based on equipment corrosion and scaling. This study demonstrated both in continuous treatment and during failure simulations that using real-time microbial monitoring meters enable more accurate microbial enumeration. As a result field operators can determine more accurate biocide dosages within 10-15 minutes of sampling. Rapid, more accurate operator responses allow companies to make better decisions and reduce operating costs.

Objective One Conclusions

Implications suggest continuous operation is the optimum running procedure for treating highly concentrated raw produced waters. Microbial activity was significantly reduced in both trials 1 and 2 during continuous filtration treatment. Significant reduction of ions was also observed when monitoring microbial electron donors and acceptors.

Overall, treated water nutrient ratios confirm that although treatment is successful in reducing nutrient levels, the overall activity potential for treated waters is still relatively high.

Objective One Recommendations

Although continuous treatment does reduce concentrations of microbial nutrient ions and scaling ions, treated waters still exhibit high reaction potentials. Dilution of raw produced waters with a small volume of freshwater would further reduce treated scaling and microbial metabolite levels contained in produced waters. Fresh water dilution volumes could be determined by calculating volumes based on 5-10% of the produced water volume needed for operations. However, since microbial levels would be increased by the combination of both fresh and produced waters, it is advised that treatment still be carried out to reduce the microbial activity prior to use. A low dose of biocide should still be added to waters before periods of storage or transport.

Objective Two Conclusions

Microbial populations appeared to re-grow over long periods of equipment downtime. A one hour downtime appeared to exhibit little change in microbial activity however, failures requiring up to four days exhibit noticeable changes in water quality

Activity in stored waters appeared to favor aerobic conditions when oxygen is the more favored electron acceptor. Although anaerobic conditions did exhibit significant growth, the level of active microbes was noticeably lower.

Objective Two Recommendations

The Failure Test implies that treatment must be continuous in order to obtain optimum results. If storage of treated water is necessary, sealed containment would be

preferred with the addition of biocide to control the slow growth of anaerobic bacteria. Anaerobic bacteria include most of the bacterial populations associated with MIC activity.[29, 55] However, if sealed containment is not possible, treatment of open air stored water must be carried out just prior to use in downhole operations to prevent microbial re-growth before use. Re-treatment of treated waters stored in sealed containment prior to use is also recommended to prevent any MIC related activity in the formation. Although treatment efforts are very successful in reducing microbial activity levels, biocide must still be administered to supplement these efforts.

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APPENDIX A

ADDITIONAL SEPARATIONS INFORMATION

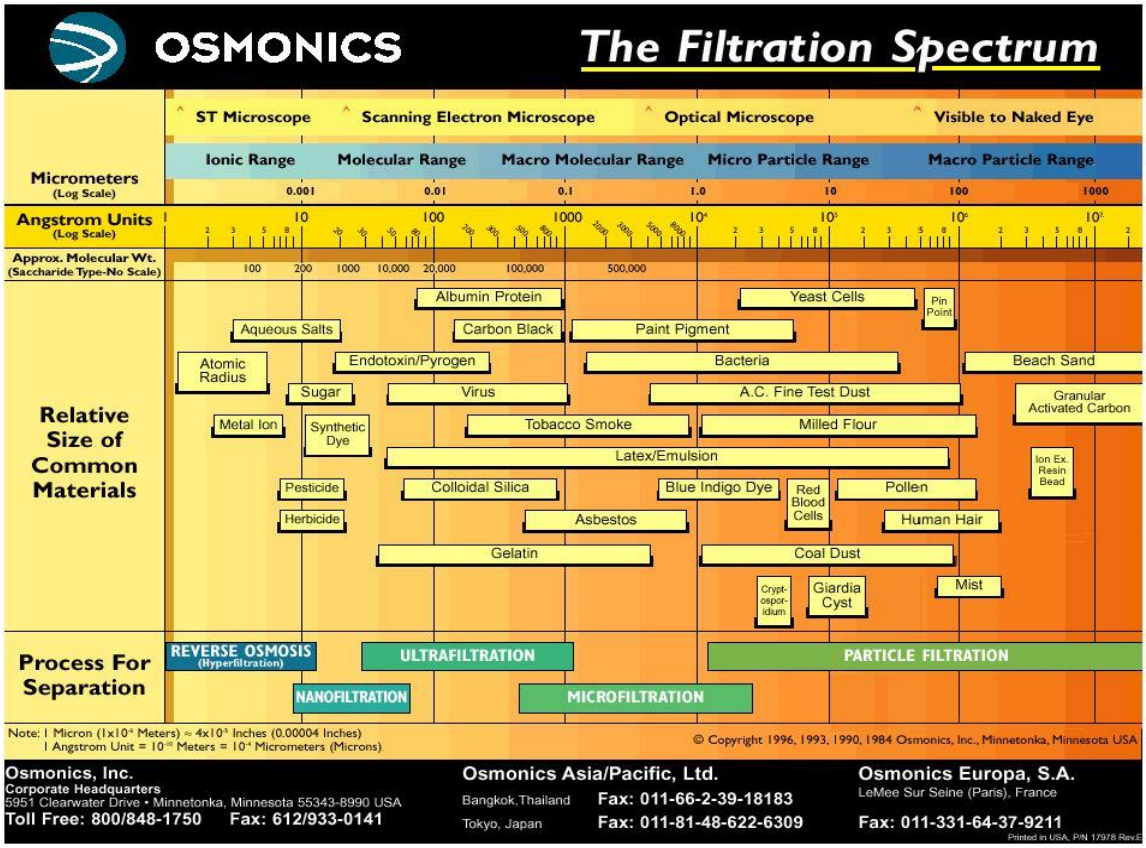

Figure A-1. Osmonics filtration spectrum describing membrane pore size and rejection capabilities.[76]

Figure A-2. Pretreatment setup for filtration feed tank, and side profile for microfiltration equipment for trials.

Figure A-3. Microfiltration (MF) equipment and computers system setup for trials.

Figure A-4. Nanofiltration (NF) equipment setup for trials. The flat sheet nanofiltration membrane is housed in a square steel housing in the middle of the table, and the polymeric nanofiltration membrane proposed for trials 2 and 3 is housed in the stainless steel cylindrical housing on top of the flat sheet housing. Stainless steel pot on the left side of the photo serves as the feed tank for the nanofiltration system.

Figure A-5. Raw produced water before pretreatment with residual oil and solids on tank wall.

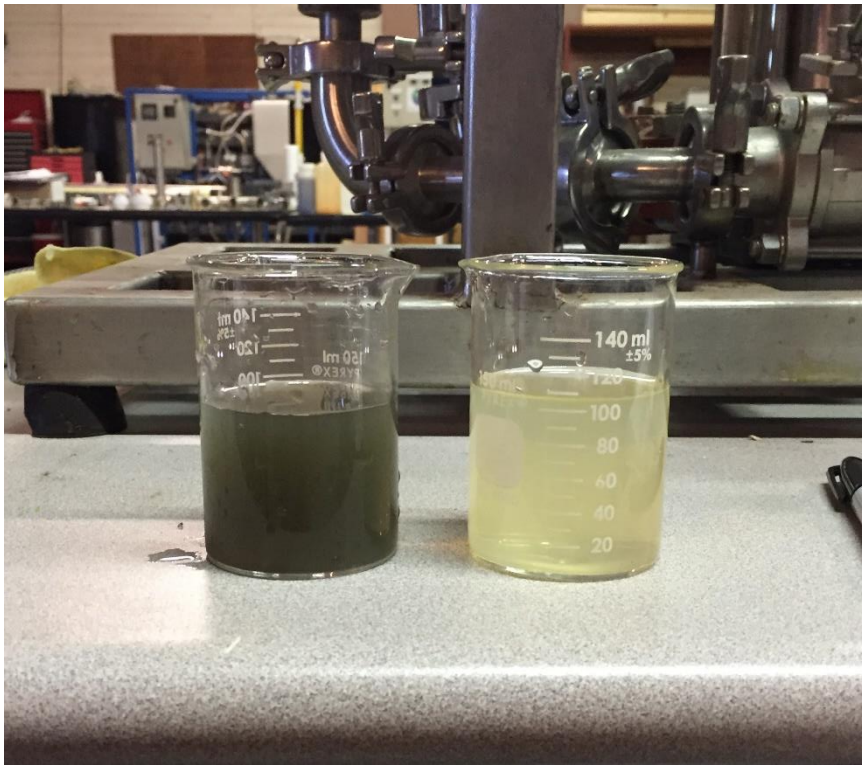

Figure A-6. Raw produced water (left) housed in beaker alongside MF treated water (right) from trial 1 to demonstrate water quality after treatment.

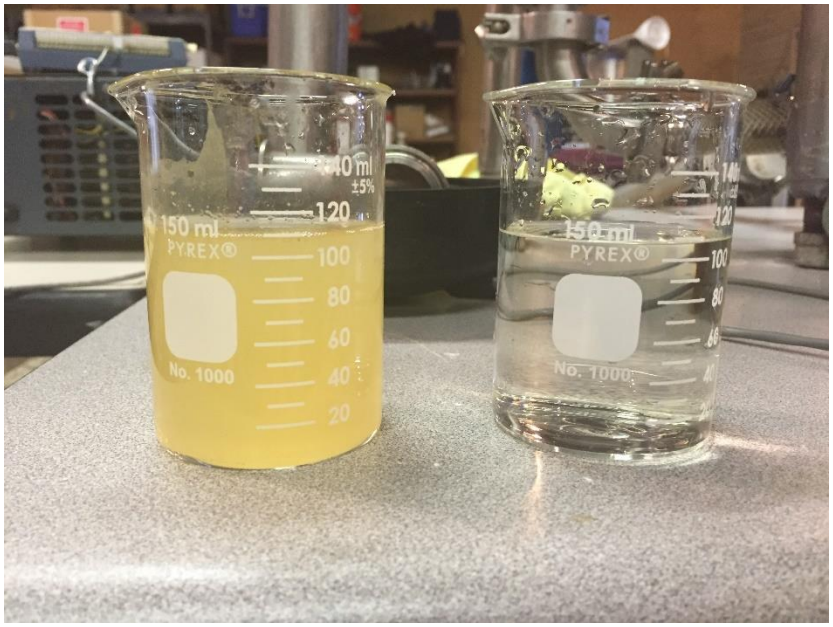

Figure A-7. MF (left) treated water housed in beaker alongside NF (right) treated water from trial 1 to demonstrate water quality after treatment.

Figure A-8. Hydraulic fracturing water storage pond at GPRI field site, Marcellus Shale.

Figure A-9. Water storage at production site, Brazos Valley area.

APPENDIX B

SUPPLEMENTAL ENCANA OIL & GAS (USA) INC. MICROBIAL STUDY

DATA

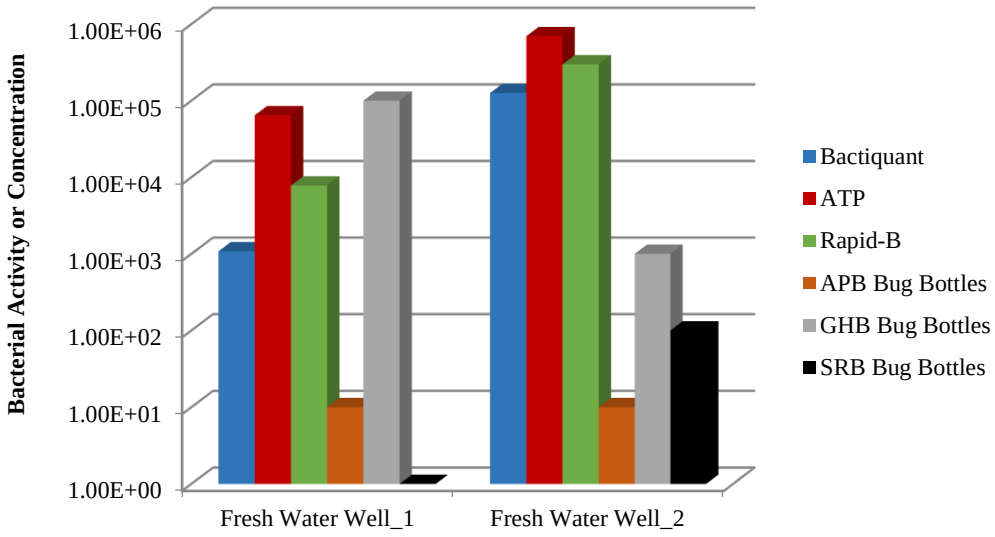

Figure B-1. Encana Oil & Gas (USA) Inc. source water wells for completion activities. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

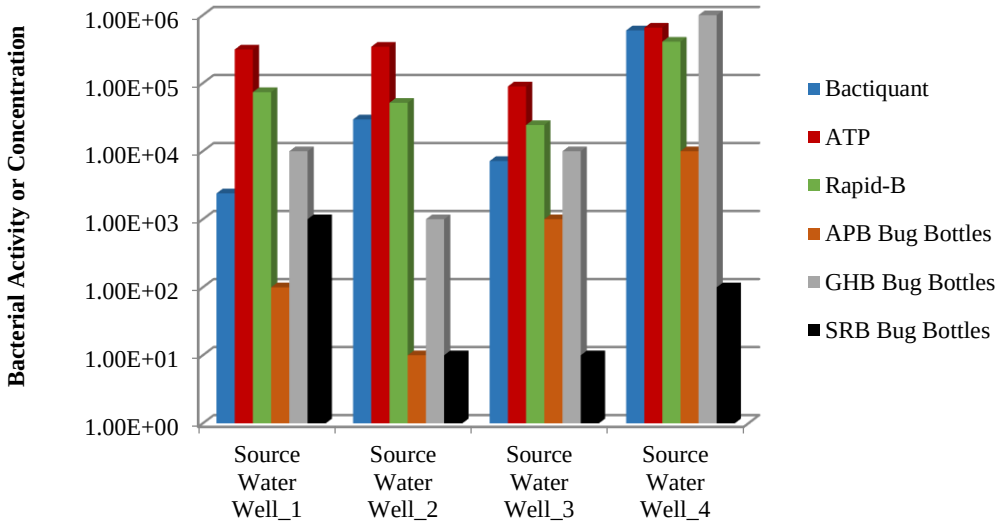

Figure B-2. Encana Oil & Gas (USA) Inc. source water wells for completion activities may contain brine water. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

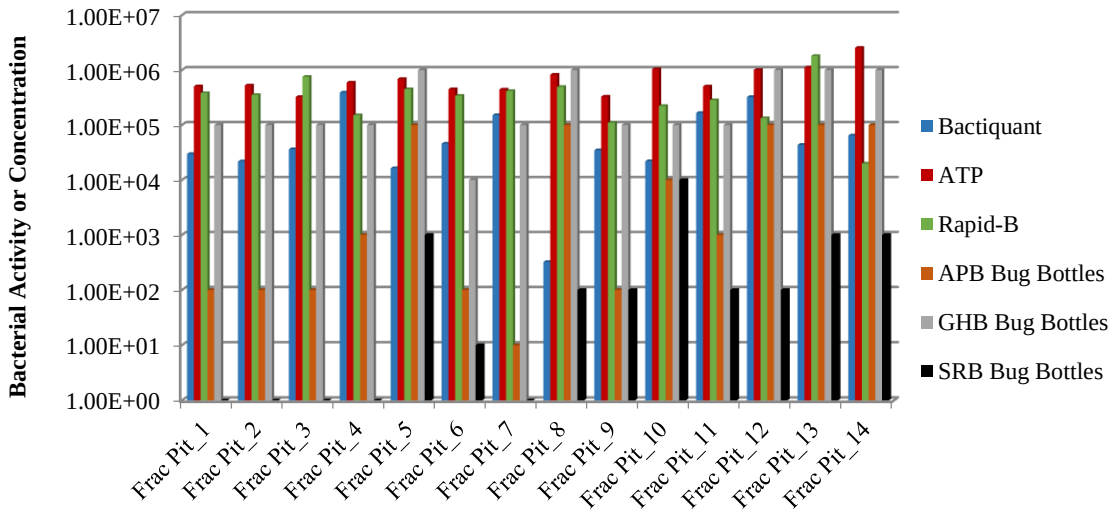

Figure B-3. Encana Oil & Gas (USA) Inc. hydraulic fracturing water pit microbial analysis for completion activities. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

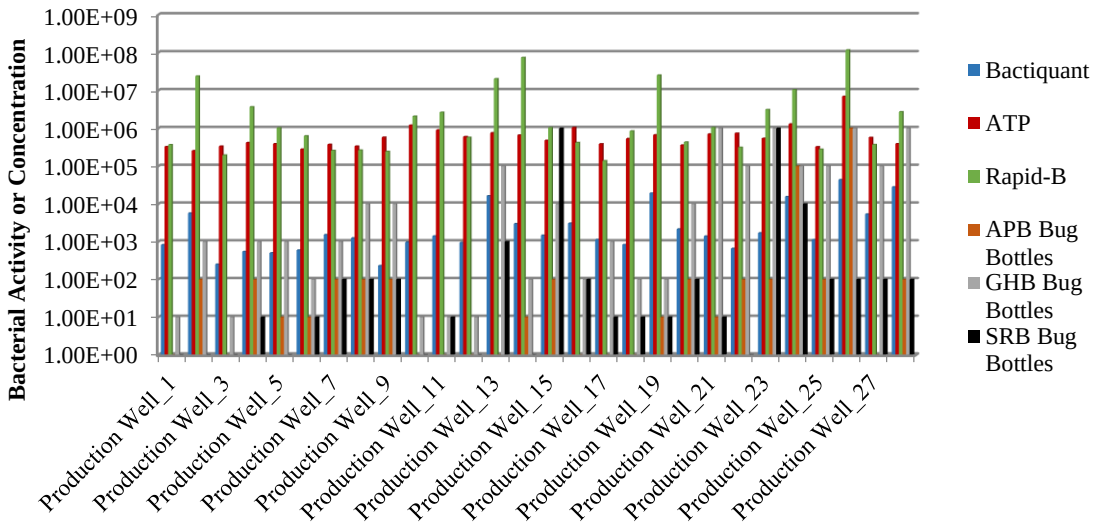

Figure B-4.. Encana Oil & Gas (USA) Inc. production wells experiencing high microbial related activity. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

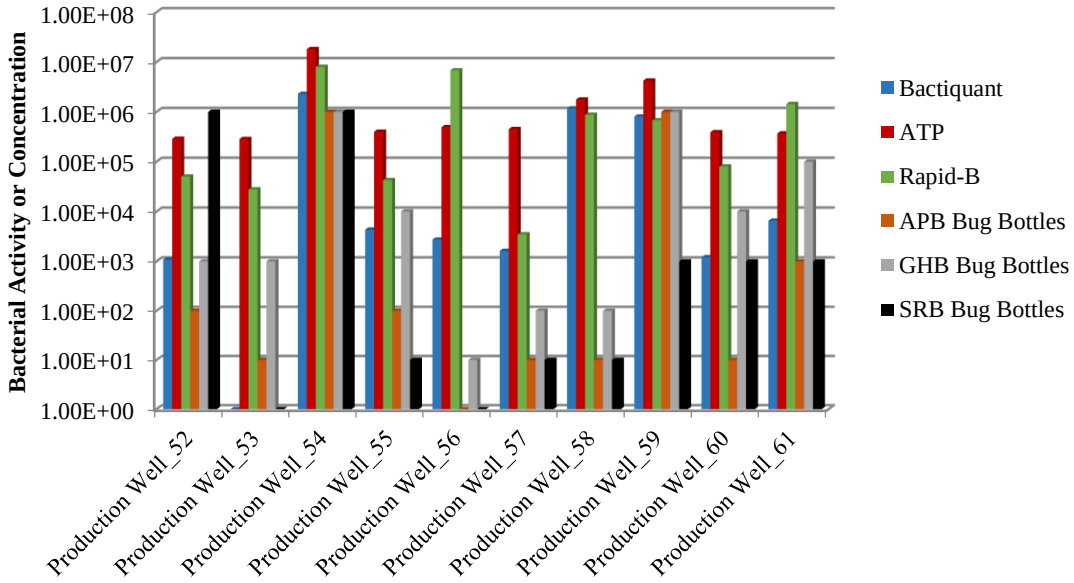

Figure B-5. Encana Oil & Gas (USA) Inc. production wells currently treated with chlorine dioxide. Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc. (Encana Oil & Gas (USA) Inc., Jennifer Fichter, 2015)

APPENDIX C

SUPPLEMENTAL ENCANA OIL & GAS (USA) INC. METAGENOMIC REPORTS

Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc.

Ecolyse, Inc.

Sample Information

Sample Count: 6 Shipped Date: 3/17/15
Shipped From: Midland, TX Arrived Date: 3/23/15

5 samples were received at Ecolyse Labs on 3/23/15. These consisted of 5 core samples. A 6th core sample was received at Ecolyse Labs on 3/25/15.

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenomic sequencing, using Ion PGM platform.

Table 1. Sample Overview

Sample	Sample Label	Ecolyse Test Requested
001	11 Mar 2015 Production Well_62	1. Metagenomics
002	06 Mar 15 Production Well_63 Sprayberry Shale	1. Metagenomics
003	3/12/15 Production Well_64_Wolfgang A	1. Metagenomics
004	Production Well_65 Bacterial Sample	1. Metagenomics
005	Production Well_66	1. Metagenomics
006	20 Mar 15 Production Well_67	1. Metagenomics

Project Results Overview: Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the sample.
- Bacterial and Archaeal diversity is determined by 16s metagenomics analysis, Ion PGM.
- Following traits assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB (sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia).
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor.
  - **APB**-(acid-producing bacteria) make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive.
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-(biodegrading bacteria) are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
- Percent of population, and number of unique microbial types (OTU) are provided as results.

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 6 samples (Table 2).
- 29111 microorganisms were analyzed genetically.
- These were grouped into 68 different microbial types (OTU).
- No Archaeal OTU were present in the samples.
- Metabolic assignments were provided for 50 of the 68 OTU's identified.
- The distribution of SRB, IRB, APB, Biodeg, and NRB is provided (Table 2, Figure 1).
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 3).
- The degree of similarity of each sample to every other sample is provided (Table 4).
- A complete list of all bacteria in the samples is available upon request.

Table 2. OG150302 Summary of Bacteria and Archaea Diversity Using Genetic Analysis
Samples are highlighted by abundances: samples highlighted in yellow have >1% metabolism of interest. Samples highlighted in grey do not have this metabolism present.

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	NRB	APB	Biodeg
OG150302-001	2000	16	None	None	None	0.6% 1 otu	None	8.2% 5 otu
OG150302-002	2637	21	None	None	None	1.21% 1 otu	None	2.77% 4 otu
OG150302-003	5547	14	None	None	None	0.018% 1 otu	None	5.79% 5 otu
OG150302-004	965	14	None	None	None	2.9% 3 otu	None	3.21% 4 otu
OG150302-005	7225	24	None	None	None	3.07% 3 otu	2.74% 1 otu	3.83% 5 otu
OG150302-006	10737	42	None	None	None	0.13% 2 otu	4.4% 5 otu	0.4% 10 otu
TOTAL	***29111***	***68***	***None***	***None***	***None***	***6 otu***	***6 otu***	***13 otu***

Table 3. Project OG150302 Metabolic Assignments of Dominant Bacterial Species

All bacterial species present in at least 1% of one sample are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundances: >10%, yellow; >1%, green; 0%, grey. A full list of all bacteria identified in these samples is available upon request.

Sample	-001	-002	-003	-004	-005	-006	Trait
Acinetobacter sp	3.2	<1%	<1%	0	0	<1%	Biodeg
Aerococcus viridans	0	0	0	0	0	9.1	GHB
Aeromonas veronii	0	1.8	2.7	2.5	3.3	<1%	Biofilm
Alkalibacterium sp	0	0	0	0	0	2.1	GHB
Alkanindiges illinoisensis	0	0	0	0	1.1	0	Biodeg
Anoxybacillus sp	<1%	0	0	0	0	68.5	Thermophile
Bacillus sp	0	0	0	0	0	5.3	Varies
Bacillus thermolactis	0	0	0	0	0	3.9	APB
Blastococcus jejuensis	0	1.6	0	0	0	0	Unknown
Brevundimonas sp	0	0	0	0	1.4	0	Biodeg
Comamonas sp	<1%	<1%	2.4	0	<1%	0	Biodeg
Comamonas testosteroni	2.5	<1%	2.9	0	<1%	0	Biodeg
Corynebacterium mucifaciens	0	0	1.3	0	0	0	GHB
Curtobacterium sp	0	5.6	0	<1%	0	0	Unknown
Cytophaga sp	1.7	0	0	0	0	0	Biodeg
Escherichia coli	81.2	69.4	83.0	88.8	69.4	3.6	Varies
Flavobacterium ceti	0	1.4	0	0	0	0	Biodeg
Halolactibacillus halophilus	0	0	0	0	0	2.3	GHB
Klebsiella sp	1	<1%	2.7	1.3	<1%	0	MIC
Micrococcus sp	0	0	0	<1%	12.0	0	GHB
Mitsuaria sp	0	0	0	0	1.7	0	Biodeg; GHB
Motilibacter peucedani	0	3.6	0	0	0.0	0	GHB
Neisseria elongata	0	1.2	0	0	0.0	0	NRB
Paracoccus sp	0	0.0	0	0	2.7	0	APB; NRB
Phaeobacter sp	0	2.4	0	0	<1%	0	GHB
Pseudoalteromonas sp	0	1.5	0	0	0	0	Biodeg
Pseudomonas balearica	1.9	<1%	0	<1%	0	0	GHB
Pseudomonas pseudoalcaligenes	<1%	0	0	1.9	0	0	Biodeg; NRB
Pseudomonas sp	<1%	1.1	0	2.1	1.0	<1%	Varies
Rickettsia typhi	0	0	3.5	0	0	0	Pathogen
Rickettsiella sp	0	0	0	0	1.4	0	Pathogen
Rubellimicrobium aerolatum	1.5	0	0	0	0	0	GHB
Schlegelella sp	0.0	2.9	0	0	0	0	Thermophile
Staphylococcus aureus	1.5	<1%	0	<1%	0	0	Pathogen
Sulfitobacter sp	0	1.6	0	0	1.1	0	SOB
Unclassified	2.0	1.3	<1%	<1%	<1%	1.4	None
Vibrio cyclitrophicus	0	1.7	0	0	0	0	GHB
Xanthomonas axonopodis	0	0	0	0	0	1.4	Unknown

Trait abbreviations:
APB, Acid-Producing Bacteria; Biodeg; Biodegradation; GHB, General Heterotrophic Bacteria; MIC, Microbial-Influenced Corrosion; NRB, Nitrogen-Reducing Bacteria; SOB, Sulfur-Oxidizing Bacteria.

Comparisons of Populations Between Samples
To get an overall view of a system, it is helpful to compare populations between different locations. Locations with similar bacterial populations reflect a combination of a common source of bacterial contamination along with a common physical environment.

Another way to present this information as a numerical value is to compare all samples to each other. This can be calculated as the number of microbes that are present in both samples as a function of all microbes present in both samples. This information is present in Table 6.

Table 4. Project OG150302 Population Similarities. Values indicate similarity of taxonomic profiles between samples. The value varies from 0 to 1, with 0 meaning that no microbiota are shared between the two samples and 1 being that all microbiota are shared between the two samples. Values of 0.5 or greater are highlighted in yellow.

OG150302	-001	-002	-003	-004	-005	-006
-001	1					
-002	0.49	1				
-003	0.40	0.46	1			
-004	0.47	0.46	0.29	1		
-005	0.40	0.40	0.42	0.42	1	
-006	0.41	0.41	0.36	0.32	0.33	1

Figure 1. Chart Showing Distribution of Select Traits Between Samples

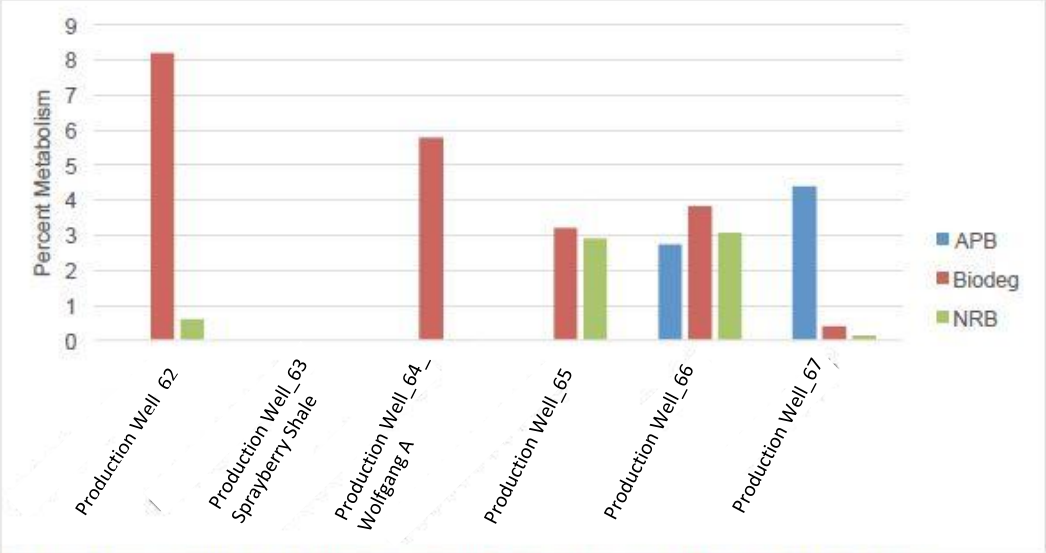

Figure 1 presents a graph with the percent abundance of key metabolic traits in each sample.

APPENDIX A. Methods

For microbial analysis, DNA was subject to bacterial tag-encoded FLX amplicon sequencing (bTEFAP) using primers 515F- GTGCCAGCMGCCGCGGTAA and
806R- TAATCTWTGGGVHCATCAGG.

Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the XXXXXXXX primer (see above primer). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the XXXXXXX primer (see above primer). Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95○C for 5 min, then 35 cycles of 94○C for 30 sec, 54○C for 40 sec, 72○C for 1 min, followed by one cycle of 72○C for 10 min and 4○C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana). Size selected pools were then quantified and 150 ng of DNA were hybridized to OT2-400 Ion Sphere beads (Life Technologies) to create single stranded DNA following Ion PGM Protocols (Life Technologies). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing following established manufacture protocols (Life Technologies).

APPENDIX B. Overview of Select Metabolic Processes

Notes on Taxonomic and Metabolic Assignment

Organisms are referred to by the identity of the most closely matched organism in the database. However, this does not indicate 100% identity. Metabolic assignments are inferred by the metabolic characteristics of the most closely related organism for which experimental data has been provided. Some metabolic groupings are overlapping and non-exclusive, e.g. many fermentative organisms generate organic acids or are capable of sulfidogenisis under some conditions.

APB: Acid-Producing Bacteria

Acid-producing bacteria are of specific interest to the oilfield community as acid production directly and aggressively promotes corrosion. Several metabolic pathways result in the production of acids, including fermentation pathways that generate organic acids such as lactic acid and acetic acid, as well as those that generate inorganic acids such as sulfuric acid as a byproduct of the oxidation of inorganic sulfur compound. It should be noted that not all fermentative pathways result in acidification of the surrounding environment. The identification of bacteria as acid producing does not necessarily indicate acidification of bulk fluids.

Biodeg: Biodegradation

Some bacterial genera and species have the capacity to utilize "atypical" or "unusual" substrates as carbon sources. These bacteria are loosely referred to as Biodeg, for "Biodegradation". The definition used here for "atypical or unusual substrates" with reference to bacterial metabolism includes compounds that most bacteria cannot utilize as a food source. Unusual compounds Biodeg organisms might be able to "eat" include disinfectants, antibiotics, xenobiotics and detergents. Some degrade long chain polymers of sugars and carbohydrates, such as those found in cell wall materials. Others are able to degrade hydrocarbons. Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons.

IRB: Iron-Reducing Bacteria, Fe(III)RB

In the absence of oxygen, many microbes can use Fe(III) as an electron acceptor, reducing it to Fe(II). Iron reduction has been observed under both acidophilic and neutrophilic conditions. Two common iron-reducing genera are Shewanella and Geobacter. In addition to IRB activity, Shewanella species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Shewanella are capable of growing in corrosive biofilms where they have been shown to remove the protective H2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. IRB should not be confused with iron oxidizing bacteria, which are aerobes responsible for a rust brown staining and slimy growth in surface waters.

NRB: Nitrate Reducing Bacteria

NRB are able to reduce nitrates to nitrites, nitrous oxide, or nitrogen under anaerobic conditions in a process termed denitrification. Most are heterotrophic facultative anaerobic bacteria including such common bacteria as *Paracoccus, Pseudomonas, Alcaligenes,* and

Bradyrhizobioium. A few bacteria use such reduction processes as hydrogen acceptor reactions and hence as a source of energy; in this case the end product is ammonia. Denitrification is a normal part of nitrogen cycling and not all NRB are of concern to O&G infrastructure.

A subcategory of NRB is the **NRSOB**: Nitrate-Reducing Sulfur-Oxidizing Bacteria are a specific subgroup of NRB whose levels are increased in reservoirs following nitrate injections (Gittel et al 2009; Grigoryan et al, 2008; Hubert and Voordouw, 2007). Growth of NRSOB suppresses the activity of SRB, and thus reducing sulfidogenisis. Some Epsilonproteobacteria can also oxidize petroleum sulfur compounds and utilize nitrate as an electron acceptor for growth, and thus may be considered hydrocarbon degrading. Massive dominance of related Epsilonproteobacteria has been observed in other petroleum samples, for example in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. (Kodama, Y and Kazuya Watanabe, 2003; Hubert et al, 2011). Sulfurospirillum are nitrate-reducing, sulfur oxidizing bacteria (NRSOB) members of the class Epsilonproteobacteria and are sometimes referred to as "Campylobacter" in older publications. The way in which nitrate addition can affect the SRB population involves several pathways. First, nitrate is a thermodynamically more favorable electron acceptor than sulfate, thus NRB have a competitive advantage. To emphasize the complexity of the metabolism in oilfield samples, it should be noted that under some conditions, these bacteria are also sulfidogens capable of reducing sulfur and thus producing H_2S (Finster K et al, 1997).

Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as "sulfidogens". Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks. It also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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Sample identifiers have been amended to adhere to confidentiality agreement with Encana Oil & Gas (USA) Inc.

Ecolyse, Inc.

Sample Information

Sample Count: 10
Shipped From: 8004 West Business 20
Midland, TX 79706

Shipped Date: 4/3/15
Arrived Date: 4/3/15

10 samples were collected by Ecolyse labs and Encana from the field on 4/3/15. These consisted of 8 liquid samples, 1 sludge sample and 1 biofilm sample.

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenomic sequencing, using Ion PGM platform.

Table 1. Sample Overview

Sample	Sample Label	Ecolyse Test Requested
001	Suction Pit Mud	1. Metagenomics
002	40	1. Metagenomics
003	25	1. Metagenomics
004	94	1. Metagenomics
005	38	1. Metagenomics
006	75	1. Metagenomics
007	68	1. Metagenomics
008	59	1. Metagenomics
009	Stan 1	1. Metagenomics
010	"Pump Jack" Sucker Rod Broken Iron Rod	1. Metagenomics

Project Results Overview: Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the sample.
- Bacterial and Archaeal diversity is determined by 16s metagenomics analysis, Ion PGM.
- The following traits were assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB (sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia).
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor.
  - **APB**-(acid-producing bacteria) these make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive.
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-biodegrading bacteria. These bacteria are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
- Percent of population, and number of unique microbial types (OTU) are provided as results.

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 10 samples (Table 2).
- One sample, OG150402-009 did not yield amplifiable DNA.
- 18694 microorganisms were analyzed genetically.
- These were grouped into 120 different microbial types (OTU).
- 2 Archaeal OTU were present in the samples.
- Metabolic assignments were provided for 92 of the 120 OTU's identified.
- The distribution of SRB, IRB, APB, Biodeg, and NRB is provided (Table 2, Figure 1).
- The degree of similarity of each sample to every other sample is provided (Table 3).
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 4).
- A complete list of all bacteria in the samples is available upon request.

Table 2. OG150401 Summary of Bacteria and Archaea Diversity Using Genetic Analysis
Samples are highlighted by abundances: samples highlighted in yellow have >1% metabolism of interest. Samples highlighted in grey do not have this metabolism present.

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	Biodeg
OG150401-001 Suction Pit Mud	8270	37	0.846% 3 otu	0.846% 3 otu	0.822% 1 otu	3.91% 3 otu	1.57% 5 otu
OG150401-002 40	377	10	32.89% 1 otu	32.89% 1 otu	None	19.36% 2 otu	None
OG150401-003 25	72	6	None	None	None	None	None
OG150401-004 94	3520	12	9.12% 1 otu	9.12% 1 otu	9.12% 1 otu	None	0.284% 1 otu
OG150401-005 38	2747	65	9.5% 1 otu	9.54% 2 otu	0.036% 1 otu	8.59% 4 otu	10.63% 8 otu
OG150401-006 75	242	13	23.14% 2 otu	24.79% 3 otu	None	None	16.53% 3 otu
OG150401-007 68	3117	35	23.55% 7 otu	13.89% 7 otu	10.75% 2 otu	0.289% 2 otu	30.57% 6 otu
OG150401-008 59	218	10	None	None	None	9.17% 1 otu	49.08% 2 otu
OG150401-009 Stan 1	None	None	None	None	None	None	None
OG150401-010	131	18	None	None	None	None	45.04% 7 otu
TOTAL	***18694***	***120***	***8 otu***	***8 otu***	***3 otu***	***9 otu***	***18 otu***

Table 3. Project OG150401 Metabolic Assignments of Dominant Bacterial Species

All bacterial species present in at least 1% of one sample are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundance: >10%, yellow; >1%, green; 0, grey. A full list of all bacteria identified in these samples is available upon request.

Species	Suction Pit Mud	40	25	94	38	75	68	59	Stan 1	Iron Rod	Trait
Acinetobacter baumannii	1.3	0	0	0	0	0	0	0	0	0	Biodeg
Actinomyces sp	0	3.2	0	0	<1	0	0	0	0	0	Ferm
Anaerophaga sp	0	0	0	0	6.1	0	17.3	0	0	0	Ferm; Thermophile
Aquimarina sp	0	0	1.4	0	0	<1	0	0	0	<1	GHB
Arcobacter sp	0	<1	0	57.5	<1	0	<1	0	0	0	NRSOB
Arthrobacter sp	0	0	0	0	0	15.3	0	0	0	0	Ferm
Atopostipes sp	0	0	0	0	1.3	0	0	0	0	0	GHB
Bordetella sp	0	0	0	0	0	0	0	0	0	6.1	Environmental
Brevibacillus sp	0	0	0	0	0	0	0	3.7	0	0	Biodeg
Brevundimonas sp	0	0	0	0	1.2	0	0	0	0	4.6	Biodeg
Butyrivibrio crossotus	1.3	0	0	0	0	0	0	0	0	0	APB; Ferm
Candidatus Arcobacter sulfidicus	0	0	0	0	<1	0	0	<1	0	1.5	NRSOB
Carnobacterium sp	0	0	0	0	0	0	0	9.2	0	0	APB; Ferm
Cellulomonas oligotrophica	0	0	0	0	0	5.4	0	0	0	0	Biodeg
Chryseobacterium hominis	0	0	0	0	1.3	0	0	0	0	0	Biodeg
Cloacibacterium sp	0	0	0	0	1.5	0	0	0	0	0	GHB
Clostridium neonatale	24.1	0	0	0	0	0	0	0	0	0	Ferm
Clostridium propionicum	2.5	0	0	0	0	0	0	0	0	0	APB
Clostridium sp	<1	0	0	<1	1.6	0	<1	0	0	0	Varies
Corynebacterium coyleae	0	0	0	0	1.7	0	0	0	0	0	GHB
Corynebacterium sp	0	<1	0	0	1.0	0	0	0	0	0	Unknown
Cytophaga sp	0	0	0	0	0	0	0	0	0	12.2	Biodeg
Delftia acidovorans	0	0	0	0	<1	9.1	<1	45.4	0	0	Biodeg; Biofilm
Desulfohalobium retbaense	0	32.9	0	0	9.5	22.3	5.2	0	0	0	SRB
Desulfohalobium utahense	0	0	0	0	0	<1	7.0	0	0	0	SRB
Desulfuromonas sp	0	0	0	0	0	0	9.8	0	0	0	IRB; SuRB
Dietzia sp	0	0	0	<1	2.9	0	0	0	0	<1	Biodeg; NRB
Flexistipes sinusarabici	0	0	0	0	0	0	2.6	0	0	0	Ferm
Fusobacterium sp	15.4	0	0	0	0	0	0	0	0	0	Anaerobe

Ecolyse, Inc.

Species	Suction Pit Mud	40	25	94	38	75	68	59	Stan 1	Iron Rod	Trait
Globicatella sulfidifaciens	2.6	0	0	0	0	0	0	0	0	0	Pathogen
Halanaerobium sp	0	0	8.3	0	<1	0	1.6	<1	0	0	Ferm; Promotes SRB
Halobacillus sp	0	0	1.4	0	0	0	3.3	0	0	0	NRB
Halolactibacillus halophilus	4.5	35.5	0	2.6	3.7	0	0	0	0	0	GHB
Halolactibacillus sp	2.2	<1	0	<1	<1	0	0	0	0	0	GHB
Halomonas neptunia	<1	0	0	0	0	0	1.1	0	0	0	Biodeg; NRB
Halomonas stenophila	0	0	0	0	0	0	2.2	0	0	0	Biodeg; NRB
Hydrogenophilus hirschii	0	0	0	0	1.1	0	0	0	0	0	H_2OX
Leifsonia lichenia	0	0	0	0	<1	1.7	0	0	0	0	GHB
Marinobacter persicus	0	0	0	0	0	0	27.7	0	0	4.6	Biodeg
Marmoricola sp	0	0	0	0	<1	0	0	1.4	0	9.9	GHB
Methanothermococcus sp	0	0	12.5	0	0	0	0	0	0	0	Methanogen; Thermophile
Methylobacterium sp	0	0	0	0	<1	0	0	14.2	0	0	Methylotroph
Mitsuaria sp	0	0	0	0	<1	2.1	<1	0	0	0	Biodeg; GHB
Mycobacterium sp	0	0	0	0	<1	0	0	0	0	3.8	Biosurfactant Producing
Nocardioides sp	0	0	0	0	0	0	0	14.7	0	9.2	Filamentous
Novosphingobium sediminicola	0	0	0	0	1.4	0	0	0	0	10.7	Biodeg
Paracoccus sp	0	4.8	0	0	0	0	0	0	0	0	APB; NRB
Pontibacter populi	0	0	0	0	2.0	0	0	0	0	0	GHB
Pseudomonas aeruginosa	0	0	0	0	1.7	0	0	0	0	0	GHB; NRB
Pseudomonas balearica	0	0	0	<1	<1	0	<1	<1	0	4.6	Biofilm; GHB
Pseudomonas fluorescens	0	0	0	0	<1	13.2	0	0	0	8.4	NRB
Pseudoxanthomonas sp	<1	0	0	0	1.6	0	0	0	0	2.3	Biodeg
Shewanella indica	<1	0	0	9.1	<1	0	0	0	0	0	IRB; MIC; SRB; TRB
Shigella flexneri	0	0	0	0	11.5	0	0	0	0	0	Pathogen
Sphingobacterium sp	0	0	0	0	<1	0	0	0	0	6.1	GHB
Sphingomonas sp	0	0	0	0	2.2	0	0	0	0	9.9	Biodeg
Sporacetigenium mesophilum	0	0	0	0	2.0	0	0	0	0	0	APB; Ferm
Staphylococcus sp	0	0	75.0	0	1.5	1.7	0	9.6	0	0	GHB

Ecolyse, Inc.

Species	Suction Pit Mud	40	25	94	38	75	68	59	Stan 1	Iron Rod	Trait
Streptococcus sp	0	14.6	0	0	4.6	0	<1	0	0	0	APB
Streptomyces sp	0	0	0	0	1.2	0	0	0	0	0	Unknown
Sulfurospirillum sp	0	0	0	0	0	0	5.2	0	0	0	NRSOB
Thermicanus aegyptius	0	0	0	0	1.8	0	0	0	0	0	APB; Thermophile
Thermoanaerobacter sp	<1	0	0	<1	<1	0	1.9	0	0	0	Ferm; Thermophile
Thermovirga sp	0	0	0	0	0	1.7	<1	0	0	0	Biofilm; SRB; SuRB; Thermophile; TRB
Unclassified	2.1	7.7	1.4	0	13.5	26.0	7.3	0	0	3.1	None
Vibrio owensii	38.4	0	0	28.4	4.6	0	<1	0	0	1.5	Biofilm; GHB

Trait abbreviations:
APB, Acid-Producing Bacteria; Biodeg; Biodegradation; IRB, Iron-Reducing Bacterial; Ferm, Fermenting Bacteria; GHB, General Heterotrophic Bacteria; H_2OX, Hydrogen-Oxidizing Bacteria; MIC, Microbial-Influenced Corrosion; NRB, Nitrogen-Reducing Bacteria; NRSOB, Nitrogen-Reducing Sulfur-Oxidizing Bacteria; SRB, Sulfate-Reducing Bacteria, SuRB, Sulfur-Reducing Bacteria; TRB, Thiosulfate-Reducing Bacteria.

Comparisons of Populations Between Samples

To get an overall view of a system, it is helpful to compare populations between different locations. Locations with similar bacterial populations reflect a combination of a common source of bacterial contamination along with a common physical environment.

Another way to present this information as a numerical value is to compare all samples to each other. This can be calculated as the number of microbes that are present in both samples as a function of all microbes present in both samples. This information is present in Table 6.

Table 4. Project OG150401 Population Similarities. Values indicate similarity of taxonomic profiles between samples. The value varies from 0 to 1, with 0 meaning that no microbiota are shared between the two samples and 1 being that all microbiota are shared between the two samples. Values of 0.5 or greater are highlighted in yellow. Values of 0 are highlighted in grey.

OG150401		-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
		Suction Pit Mud	40	25	94	38	75	68	59	Stan 1	Iron Rod
-1	Suction Pit Mud	1									
-2	40	0.09	1								
-3	25	0	0	1							
-4	94	0.25	0.29	0	1						
-5	38	0.24	0.19	0.06	0.26	1					
-6	75	0	0.10	0.24	0	0.16	1				
-7	68	0.20	0.19	0.10	0.22	0.22	0.26	1			
-8	59	0	0	0.27	0.09	0.19	0.18	0.14	1		
-9	Stan 1	0	0	0	0	0	0	0	0	0	
-10	Iron Rod	0.08	0	0.09	0.21	0.30	0.14	0.12	0.30	0	1

Figure 1. Chart Showing Distribution of Select Traits Between Samples

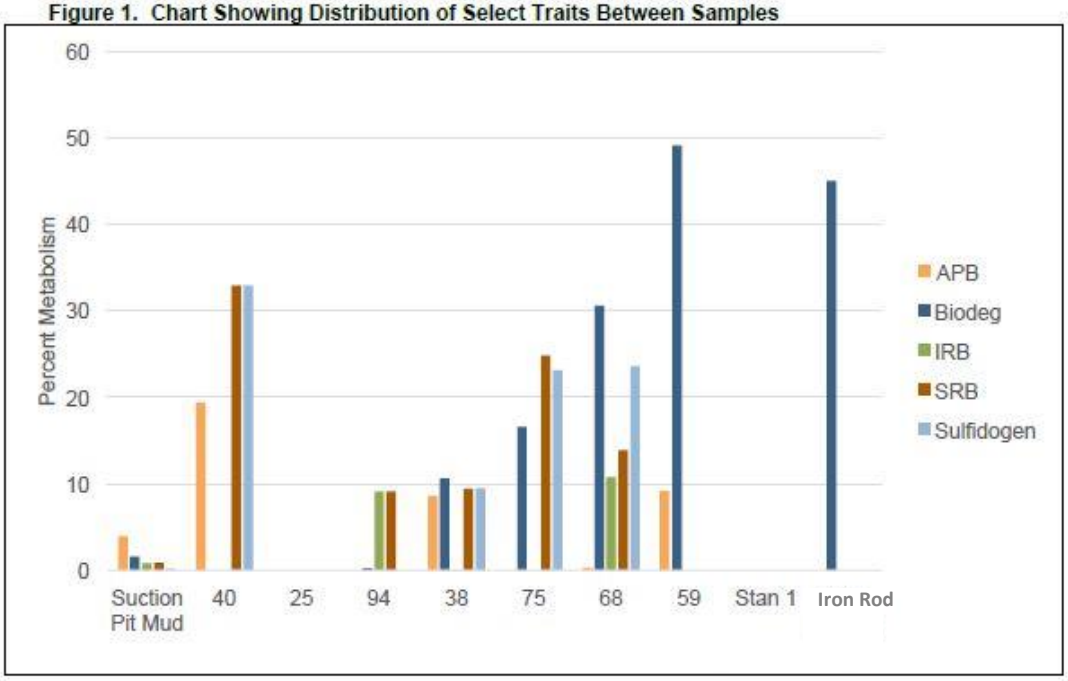

Figure 1 presents a graph with the percent abundance of key metabolic traits in each sample.

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APPENDIX B. Overview of Select Metabolic Processes

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Biodeg: Biodegradation

Some bacterial genera and species have the capacity to utilize "atypical" or "unusual" substrates as carbon sources. These bacteria are loosely referred to as Biodeg, for "Biodegradation". The definition used here for "atypical or unusual substrates" with reference to bacterial metabolism includes compounds that most bacteria cannot utilize as a food source. Unusual compounds Biodeg organisms might be able to "eat" include disinfectants, antibiotics, xenobiotics and detergents. Some degrade long chain polymers of sugars and carbohydrates, such as those found in cell wall materials. Others are able to degrade hydrocarbons. Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons.

IRB: Iron-Reducing Bacteria, Fe(III)RB

In the absence of oxygen, many microbes can use Fe(III) as an electron acceptor, reducing it to Fe(II). Iron reduction has been observed under both acidophilic and neutrophilic conditions. Two common iron-reducing genera are Shewanella and Geobacter. In addition to IRB activity, Shewanella species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Shewanella are capable of growing in corrosive biofilms where they have been shown to remove the protective H2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. IRB should not be confused with iron oxidizing bacteria, which are aerobes responsible for a rust brown staining and slimy growth in surface waters.

NRB: Nitrate-Reducing Bacteria

NRB are able to reduce nitrates to nitrites, nitrous oxide, or nitrogen under anaerobic conditions in a process termed denitrification. Most are heterotrophic facultative anaerobic bacteria including such common bacteria as *Paracoccus, Pseudomonas, Alcaligenes,* and

Bradyrhizobioium. A few bacteria use such reduction processes as hydrogen acceptor reactions and hence as a source of energy; in this case the end product is ammonia. Denitrification is a normal part of nitrogen cycling and not all NRB are of concern to O&G infrastructure.

A subcategory of NRB is the **NRSOB**: Nitrate-Reducing Sulfur-Oxidizing Bacteria are a specific subgroup of NRB whose levels are increased in reservoirs following nitrate injections (Gittel et al 2009; Grigoryan et al, 2008; Hubert and Voordouw, 2007). Growth of NRSOB suppresses the activity of SRB, and thus reducing sulfidogenisis. Some Epsilonproteobacteria can also oxidize petroleum sulfur compounds and utilize nitrate as an electron acceptor for growth, and thus may be considered hydrocarbon degrading. Massive dominance of related Epsilonproteobacteria has been observed in other petroleum samples, for example in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. (Kodama, Y and Kazuya Watanabe, 2003; Hubert et al, 2011). Sulfurospirillum are nitrate-reducing, sulfur oxidizing bacteria (NRSOB) members of the class Epsilonproteobacteria and are sometimes referred to as "Campylobacter" in older publications. The way in which nitrate addition can affect the SRB population involves several pathways. First, nitrate is a thermodynamically more favorable electron acceptor than sulfate, thus NRB have a competitive advantage. To emphasize the complexity of the metabolism in oilfield samples, it should be noted that under some conditions, these bacteria are also sulfidogens capable of reducing sulfur and thus producing H_2S (Finster K et al, 1997).

Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as "sulfidogens". Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks. It also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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Ecolyse, Inc.

Sample Information

Sample Count: 4
Shipped From: Midland, TX

Shipped Date: 5/1/15
Arrived Date: 5/5/15

4 samples were received at Ecolyse Labs on 5/5/15. These consisted of 4 liquid samples.

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenomic sequencing, using Ion PGM platform.

TABLE 1. Sample Overview

Sample	Sample Label	Ecolyse Test Requested	DNA ng/mL	Bacterial cells/mL**
001	Drilling Mud 1	1. Metagenomics	11	2.44E+08
002	Drilling Mud 2	1. Metagenomics	5.6	1.24E+08
003	Drilling Mud 3	1. Metagenomics	5.8	1.29E+08
004	Drilling Mud 4	1. Metagenomics	0	0
**Assumes 3.3 fg DNA/cell				

Project Results Overview: Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the sample.
- Bacterial and Archaeal diversity is determined by 16s metagenomics analysis, Ion PGM.
- Following traits assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB(sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia)
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor
  - **APB**-(acid-producing bacteria) these make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-biodegrading bacteria. These bacteria are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
- Percent of population, and number of unique microbial types (OTU) are provided as results

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 8 samples (Table 1).
- One sample (Drilling Mud 4) did not yield DNA.
- One sample (Drilling Mud 1) yielded DNA but it was unable to be amplified.
- 23897 microorganisms were analyzed genetically.
- These were grouped into 76 different microbial types (OTU).
- 1 Archaeal OTU were present in the samples.
- Metabolic assignments were provided for 57 of the 76 OTU's identified.
- The distribution of SRB, IRB, APB, Biodeg, and NRB is provided (Table 2, Figure 1).
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 3).
- The degree of similarity of each sample to every other sample is provided.
- A complete list of all bacteria in the samples is available upon request.

Table 2. OG150501 Summary of Bacteria and Archaea Diversity Using Genetic Analysis

Samples are highlighted by abundance: samples highlighted in yellow have >1% metabolism of interest. Samples highlighted in grey do not have this metabolism present.

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	Biodeg	NRB
OG150501-002 Drilling Mud 2	18642	63	34% 7 OTU	32.17% 3 OTU	5.3% 4 OTU	0.719% 2 OTU	31.52% 12 OTU	35.1% 11 OTU
OG150501-003 Drilling Mud 3	5255	37	16.67% 2 OTU	0.057% 1 OTU	None	6.41% 3 OTU	6.95% 5 OTU	8.94% 7 OTU
TOTAL	*23897*	*76*	*8 OTU*	*4 OTU*	*4 OTU*	*3 OTU*	*13 OTU*	*12 OTU*

Table 3. Project OG150501 Metabolic Assignments of Dominant Bacterial Species

All bacterial species present in at least 1% of one sample are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundance: >10%, yellow; >1%, green; 0, grey. A full list of all bacteria identified in these samples is available upon request.

Species	Drilling Mud 2	Drilling Mud 3	Trait
Aeromonas sp	0	8.9	Biofilm
Alishewanella sp	<1	15.1	Sulfidogen; TRB
Anoxybacillus sp	<1	8.4	Thermophile
Arcobacter sp	3.2	0	NRSOB
Bacteroides graminisolvens	0	11.0	Ferm
Bacteroides sp	<1	9.7	Ferm
Clostridium propionicum	0	1.3	APB
Clostridium sp	<1	2.0	Varies
Desulfocella sp	31.0	0	SRB; Sulfidogen
Desulfuromonas sp	1.7	0	IRB; Sulfidogen; SuRB
Fusibacter tunisiensis	0	1.6	Sulfidogen; SuRB; TRB
Halolactibacillus halophilus	2.8	8.8	Alkaliphile; Halophile
Halolactibacillus miurensis	<1	9.7	Alkaliphile; Halophile
Halomonas sp	16.7	5.8	Biodeg (HC); NRB
Marinifilum sp	2.5	0	Filamentous
Marinilabilia sp	6.3	0	Biodeg
Marinobacter sp	2.2	<1	Biodeg (HC); NRB
Marinobacterium sp	4.8	0	Biodeg (HC); NRB
Pelobacter sp	3.5	0	IRB
Pseudomonas sp	<1	2.6	Varies
Thiomicrospira thermophila	1.2	0	NRSOB; Thermophile
Trichococcus sp	<1	4.9	APB; Ferm
Unclassified	10.0	2.9	None
Vibrio diabolicus	5.5	2.4	Biofilm; NRB

Trait abbreviations:

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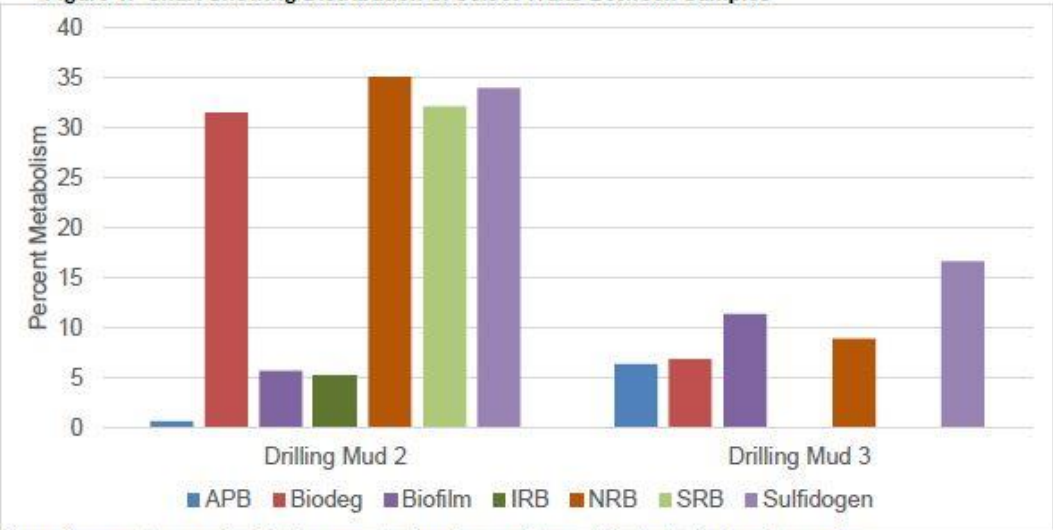
Another way to present this information as a numerical value is to compare all samples to each other. This can be calculated as the number of microbes that are present in both samples as a function of all microbes present in both samples. Samples showed a comparison value of 0.48, meaning the samples had 48% of microbial taxa present in both samples.

Figure 1. Chart Showing Distribution of Select Traits Between Samples



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Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as "sulfidogens". Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks, it also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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APPENDIX D

SUPPLEMENTAL MICROBIAL GROWTH DATA FROM FILTRATION ANALYSIS

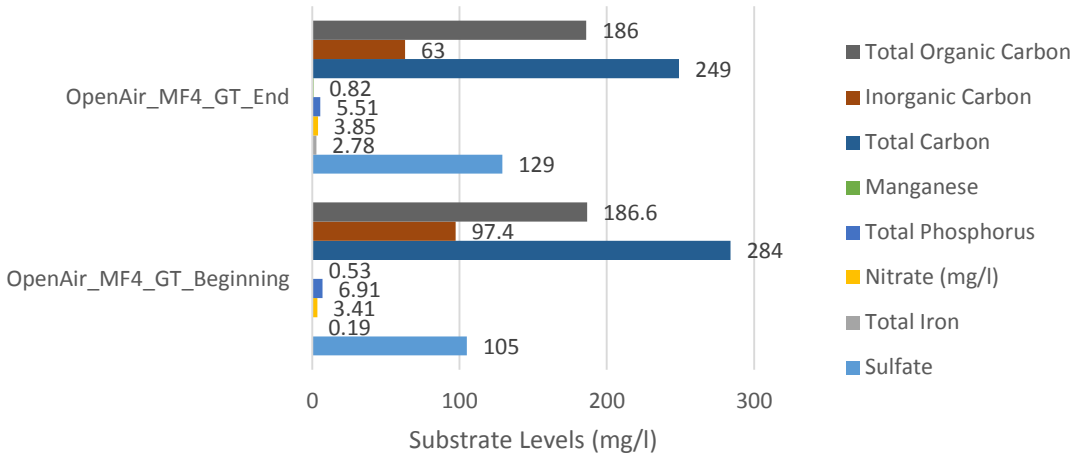

Figure D-1. Microbial substrate levels post four day open air growth analysis.

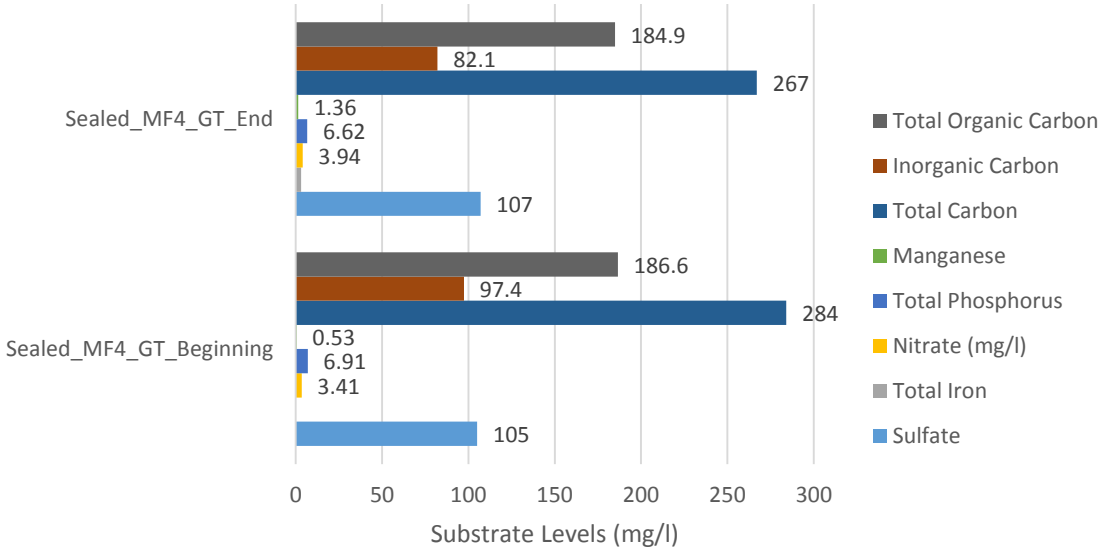

Figure D-2. Microbial substrate levels post four day sealed growth analysis.

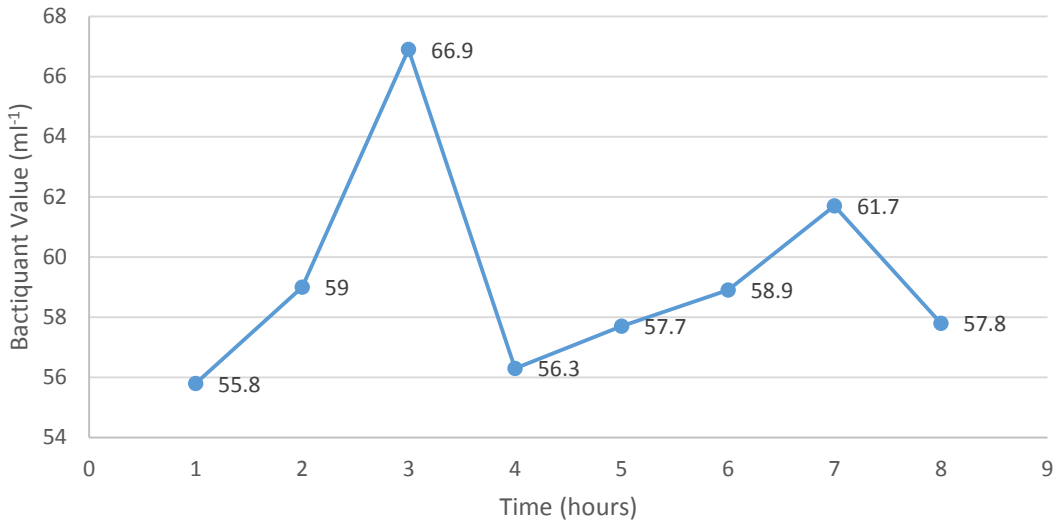

Figure D-3. Eight hour growth analysis of MF permeate in sealed container.

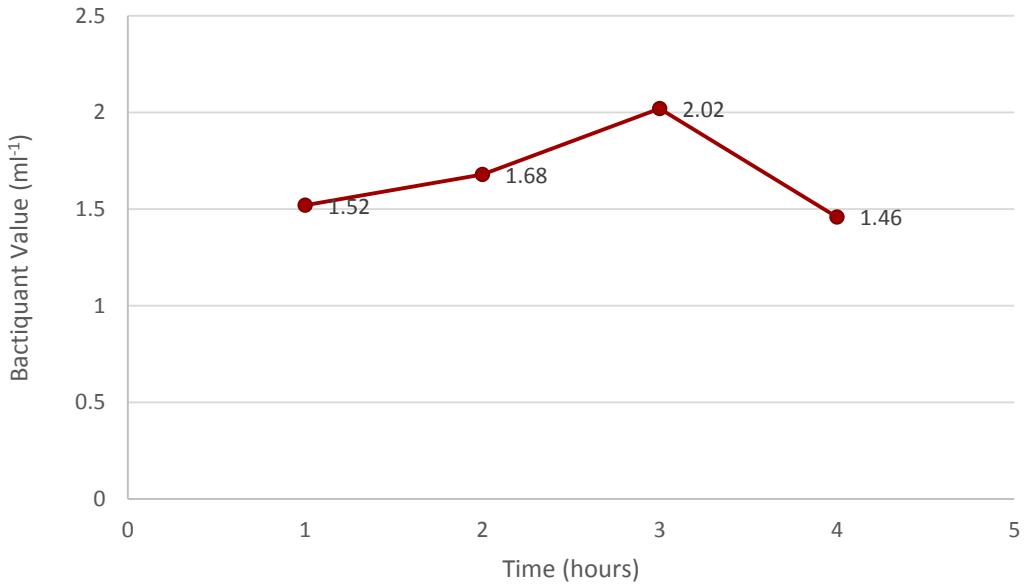

Figure D-4. Eight hour growth analysis of NF permeate in sealed container.

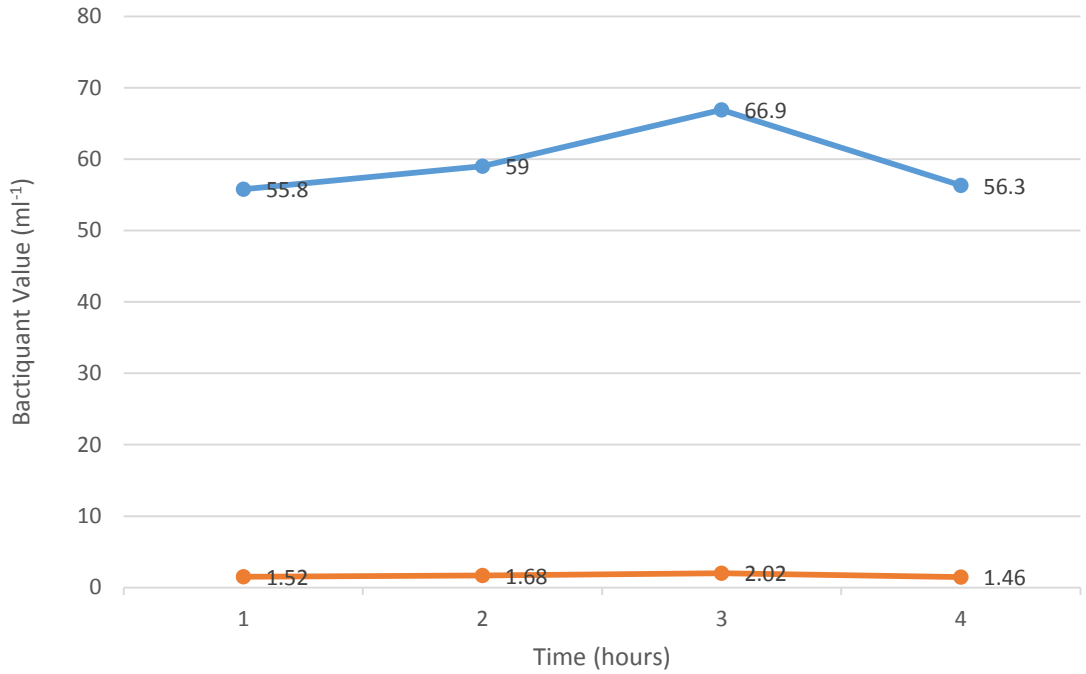

Figure D-5. Eight hour growth analysis of MF and NF permeates in sealed containers.

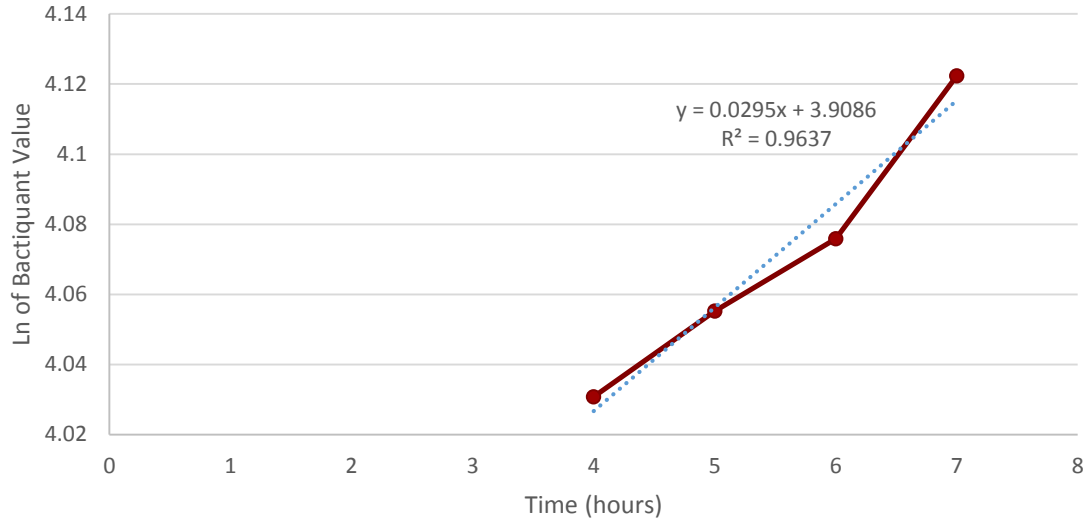

Figure D-6. Natural log of eight hour growth analysis of MF permeate in sealed containers. Data was taken from the most linear section of the original growth analysis to develop a linear rate equation to calculate the maximum net specific growth rate of bacteria.

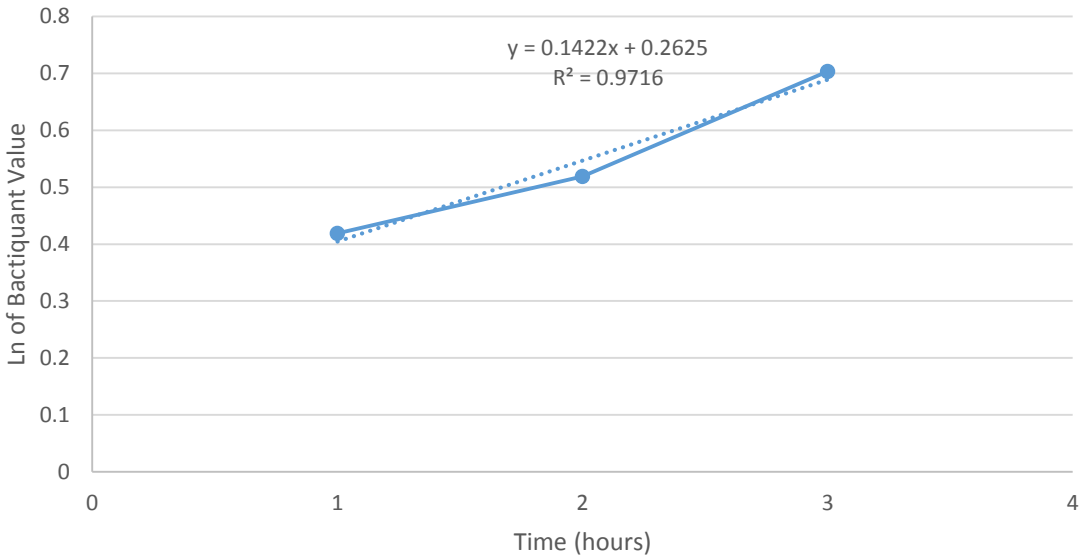

Figure D-7. Natural log of eight hour growth analysis of NF permeate in sealed containers. Data was taken from the most linear section of the original growth analysis to develop a linear rate equation to calculate the maximum net specific growth rate of bacteria.

APPENDIX E

SUPPLEMENTAL MICROBIAL NUTRIENT DATA FROM FILTRATION ANALYSIS

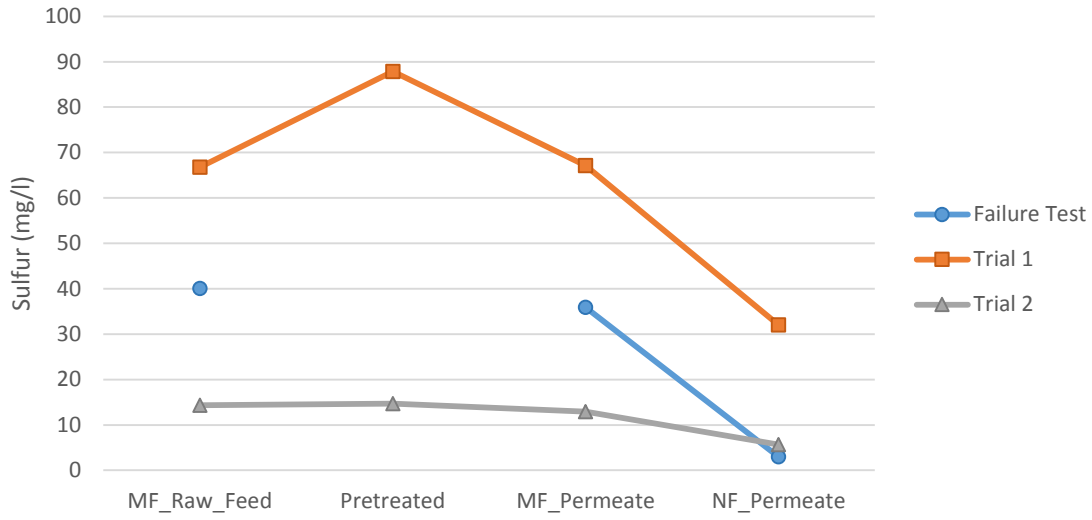

Figure E-1. Total elemental sulfur post treatment with MF and NF technologies.

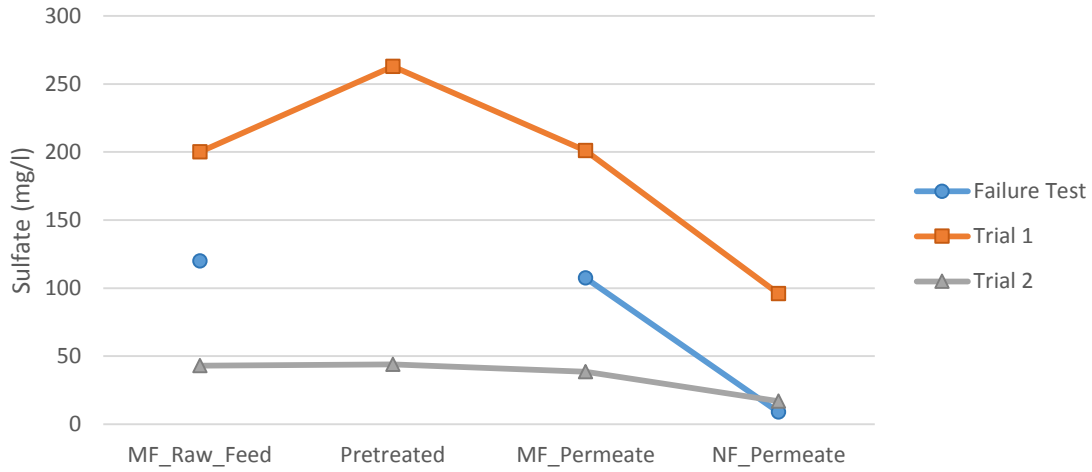

Figure E-2. Sulfate levels post treatment with MF and NF technologies.

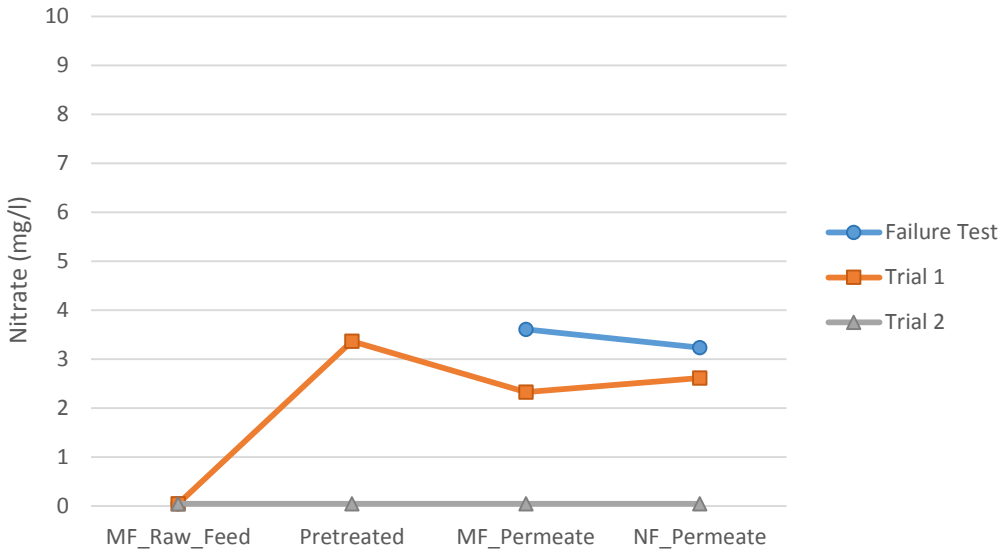

Figure E-3. Nitrate levels post treatment with MF and NF technologies.

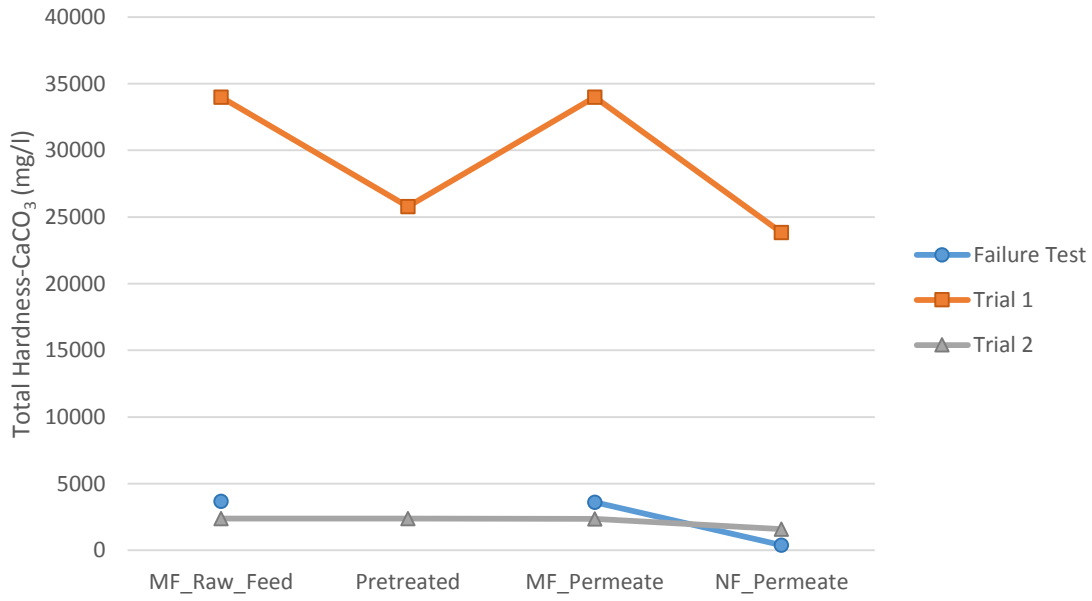

Figure E-4. Total hardness post treatment with MF and NF technologies.

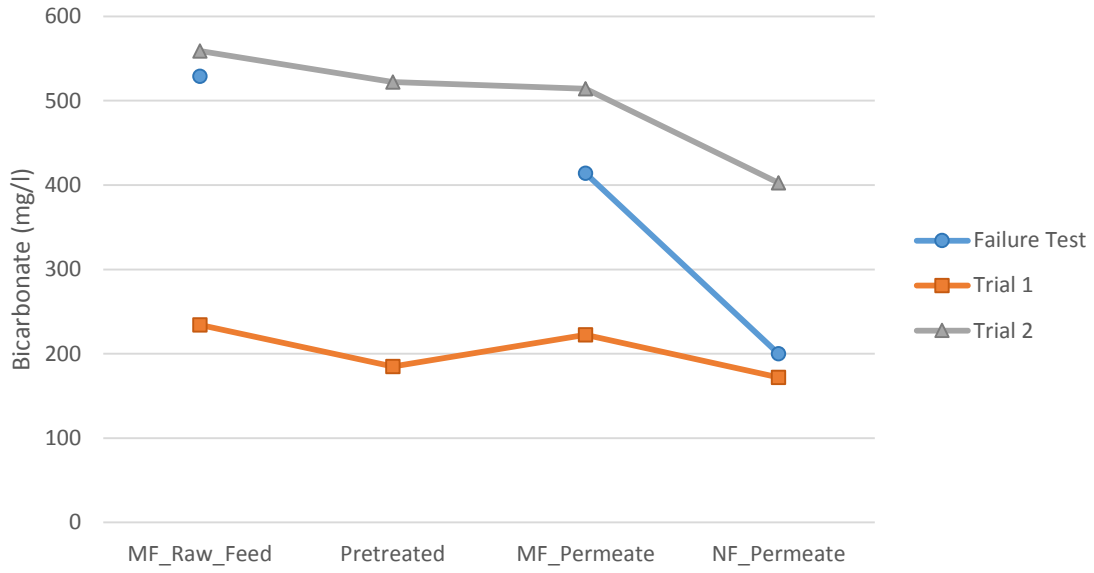

Figure E-5. Bicarbonate levels post treatment with MF and NF technologies.

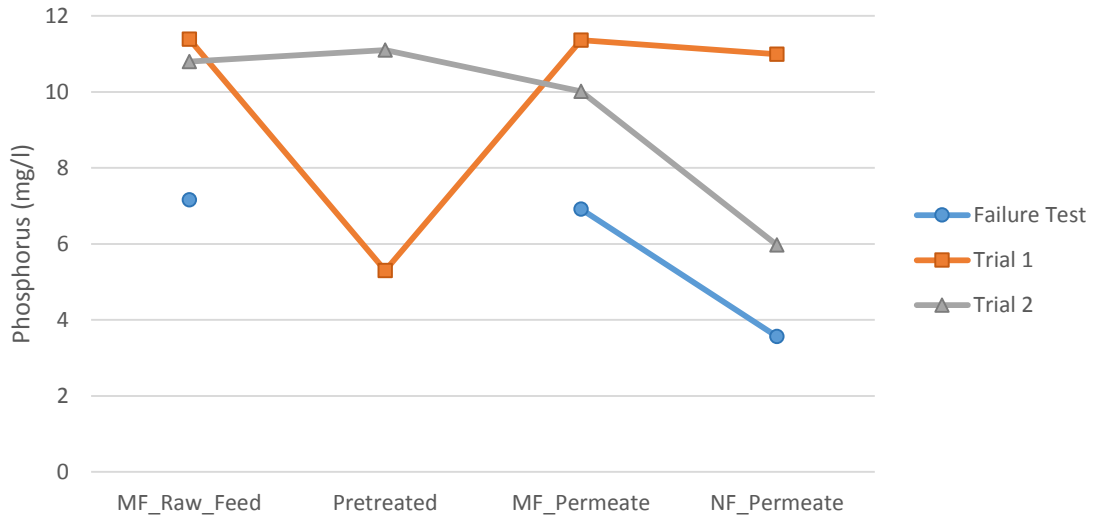

Figure E-6. Total elemental phosphorus post treatment with MF and NF technologies.

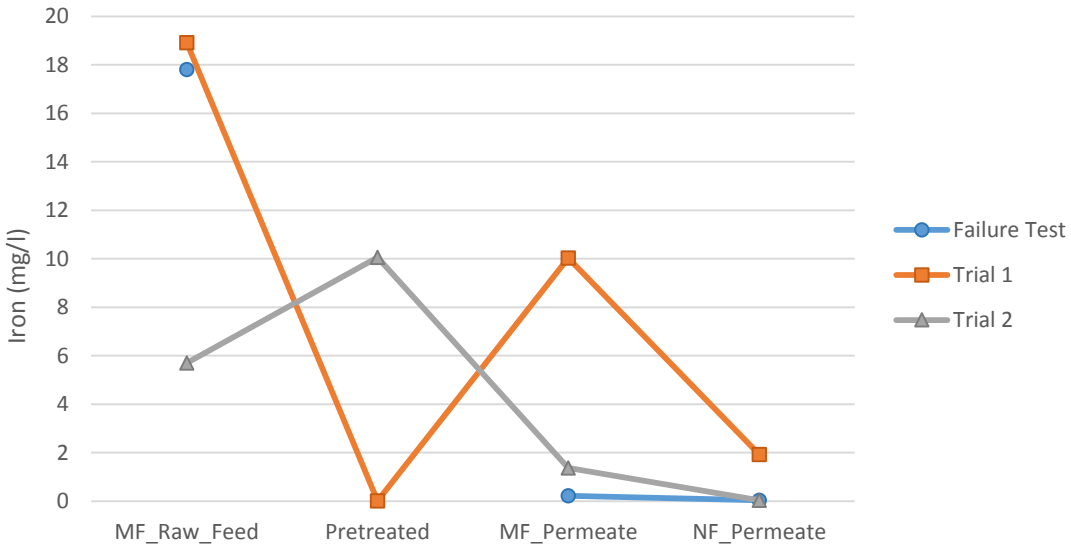

Figure E-7. Total soluble iron post treatment with MF and NF technologies.

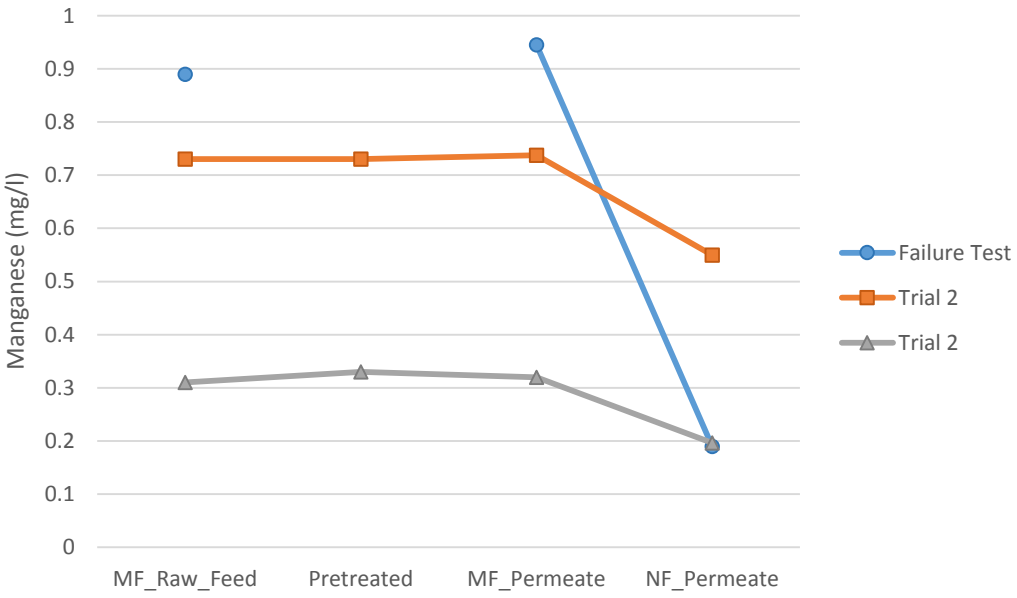

Figure E-8. Manganese levels post treatment with MF and NF technologies.

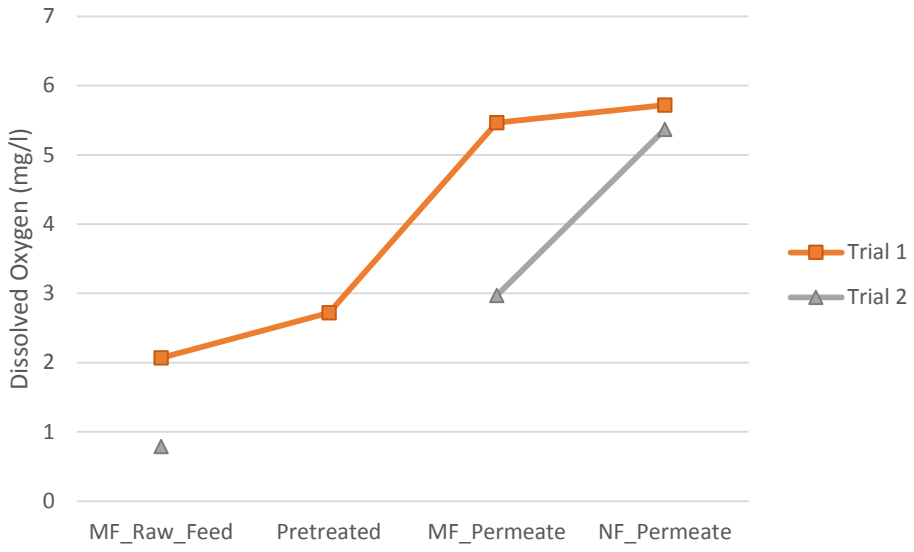

Figure E-9. Dissolved oxygen post treatment with MF and NF technologies.

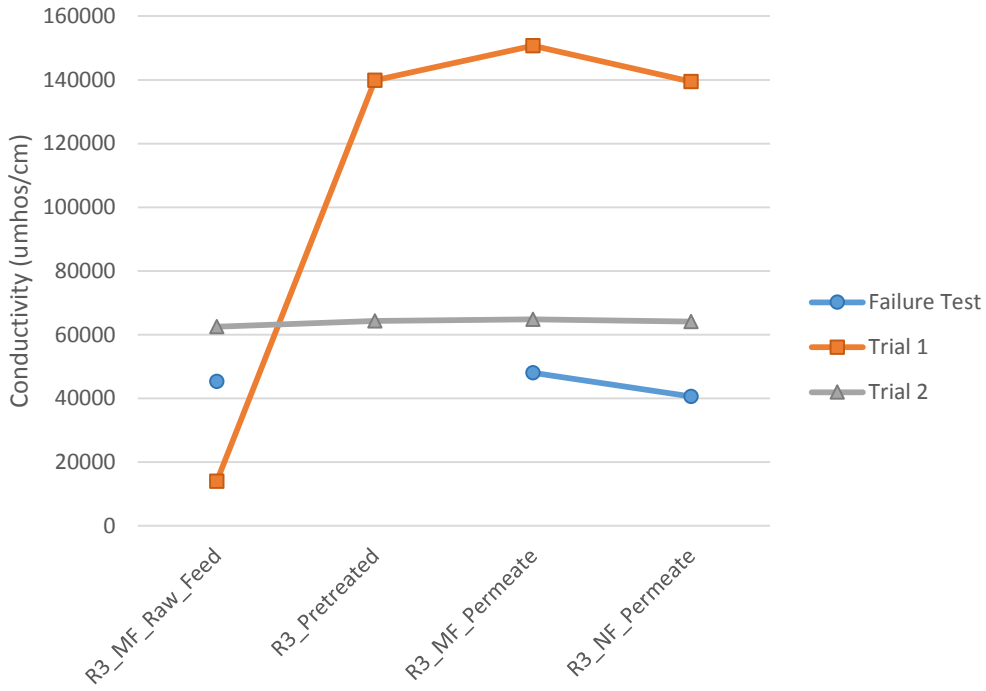

Figure E-10. Conductivity post treatment with MF and NF technologies.

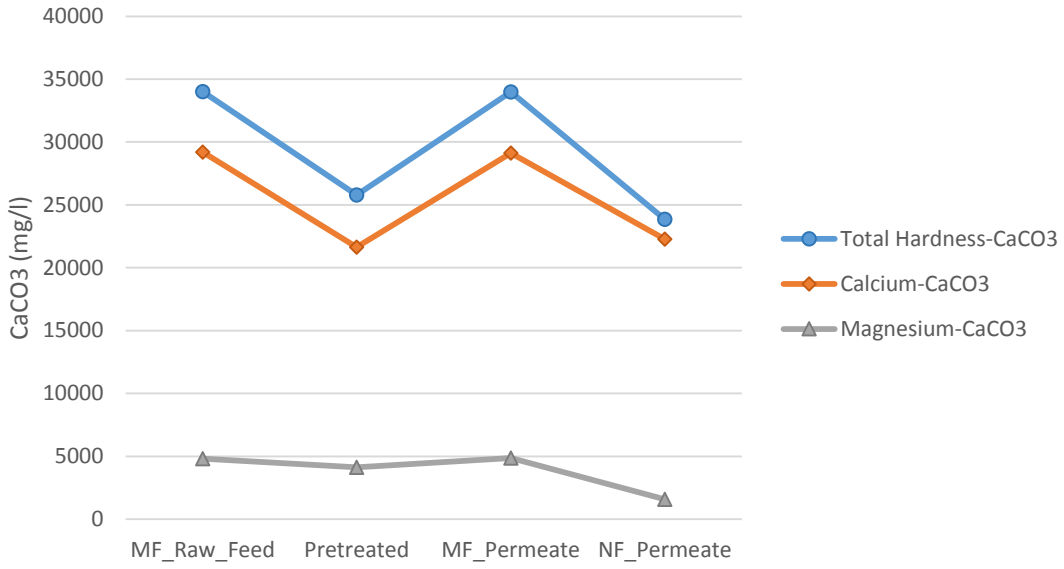

Figure E-11. Total hardness post treatment with MF and NF technologies in trial 1.

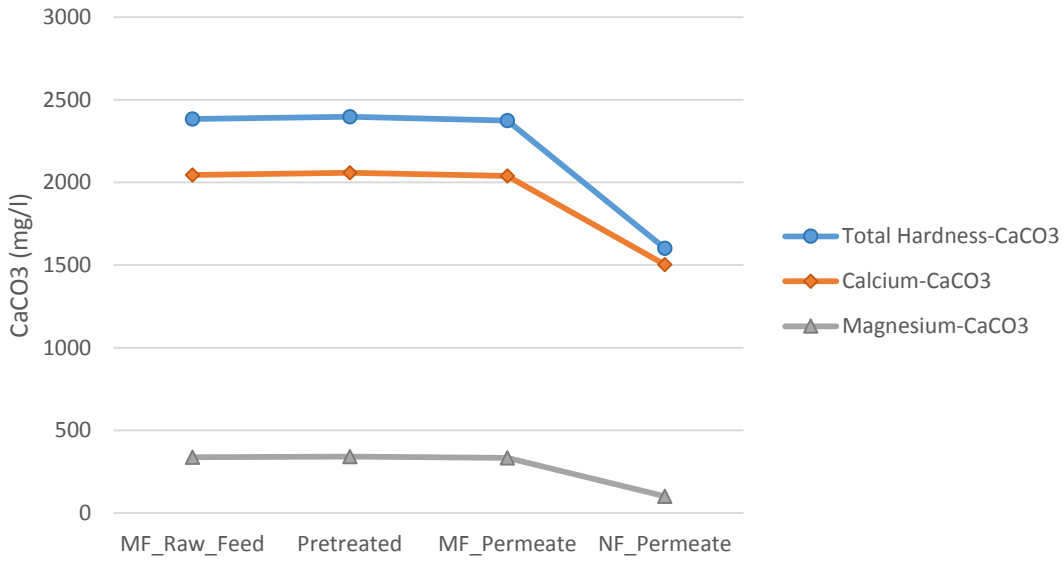

Figure E-12. Total hardness post treatment with MF and NF technologies in trial 2.

APPENDIX F

SUPPLEMENTAL RAW PRODUCED WATER METAGENOMIC DATA FROM FILTRATION ANALYSIS

Ecolyse, Inc.

Sample Information

Sample Count: 3
Shipped From: 407 Richardson Building 3116
College Station, Texas

Shipped Date: 08/06/2015
Arrived Date: 08/06/2015

3 samples were received at Ecolyse Labs on 8/6/15. These consisted of 3 liquid samples.

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenomic sequencing, using Ion PGM platform.

TABLE 1. Sample Overview

Sample	Sample Label	Ecolyse Test Requested	DNA ng/mL	Bacterial cells/mL*
001	Raw Feed	1.Metagenomics	3.90	8.12E+06
002	MF_Permeate 1	1.Metagenomics	ND	ND
003	MF_Permeate 4	1.Metagenomics	ND	ND
****Assumes 3.3 fg DNA/cell ND-no data as no DNA was isolated**				

Project Results Overview: Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the sample.
- Bacterial and Archaeal diversity is determined by 16s metagenomics analysis, Ion PGM.
- Following traits assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB(sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia)
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor
  - **APB**-(acid-producing bacteria) these make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-biodegrading bacteria. These bacteria are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
- Percent of population, and number of unique microbial types (OTU) are provided as results

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 3 samples (Table 1).
  - No DNA was isolated from samples OG150802-002 and OG150803-003
- 31281 microorganisms were analyzed genetically.
- These were grouped into 57 different microbial types (OTU).
- 1 Archaeal OTU were present in the samples.
- Metabolic assignments were provided for 43 of the 57 OTU's identified.
- The distribution of SRB, IRB, APB, Biodeg, and NRB is provided (Table 2, Figure 1).
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 3).
- A complete list of all bacteria in the samples is provided (Table 4).

Table 2. OG150802 Summary of Bacteria and Archaea Diversity Using Genetic Analysis

Samples are highlighted by abundances: samples highlighted in yellow have >1% metabolism of interest. Samples highlighted in grey do not have this metabolism present.

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	Biodeg	NRB
OG150802-001 Raw Feed	31281	57	18.5% 9 OTU	18.5% 9 OTU	1.47% 2 OTU	1.06% 4 OTU	None	None
TOTAL	***31281***	***57***	***9 OTU***	***9 OTU***	***2 OTU***	***4 OTU***	***None***	***None***

Table 3. Project OG150802 Metabolic Assignments of Dominant Bacterial Species

All bacterial species present in at least 1% of one sample are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundances: >10%, yellow; >1%, green; 0, grey.

Species	OG150802-001 Raw Feed	Trait
Bacillus weihenstephanensis	2.727	GHB
Clostridiisalibacter paucivorans	1.295	Homoacetogen
Clostridium sp	4.114	Varies
Desulfovibrio alaskensis	14.469	Oilfield; SRB, Sulfidogen
Desulfovibrio sp	1.723	SRB; Sulfidogen
Geosporobacter sp	1.435	IRB
Oceanotoga sp	1.461	SRB; SuRB; TRB
Salinivibrio sp	37.01	GHB
Unclassified	31.175	None

Trait abbreviations:
IRB, Iron-Reducing Bacterial; GHB, General Heterotrophic Bacteria; SRB, Sulfate-Reducing Bacteria, SuRB, Sulfur-Reducing Bacteria; TRB, Thiosulfate-Reducing Bacteria.

Table 4. Total Species List for project OG150802.

Class	Species	OG150802-001
		Raw Feed
Gammaproteobacteria	*Acinetobacter sp*	0.115
Gammaproteobacteria	*Alteromonas macleodii*	0.016
Synergistia	*Aminomonas sp*	0.019
Bacteroidia	*Anaerophaga sp*	0.118
Clostridia	*Anaerovorax sp*	0.205
Epsilonproteobacteria	*Arcobacter halophilus*	0.307
Gammaproteobacteria	*Arhodomonas sp*	0.035
Bacilli	*Bacillus cereus*	0.038
Bacilli	*Bacillus sp*	0.006
Bacilli	*Bacillus vireti*	0.026
Bacilli	*Bacillus weihenstephanensis*	2.727
Clostridia	*Caldanaerocella sp*	0.013
Clostridia	*Caminicella sp*	0.064
Clostridia	*Clostridiisalibacter paucivorans*	1.295
Clostridia	*Clostridium halophilum*	0.125
Clostridia	*Clostridium sp*	4.114
Deltaproteobacteria	*Desulfomicrobium apsheronum*	0.537
Deltaproteobacteria	*Desulfomicrobium sp*	0.083
Deltaproteobacteria	*Desulfovibrio alaskensis*	14.469
Deltaproteobacteria	*Desulfovibrio caledoniensis*	0.032
Deltaproteobacteria	*Desulfovibrio capillatus*	0.176
Deltaproteobacteria	*Desulfovibrio marinus*	0.01
Deltaproteobacteria	*Desulfovibrio sp*	1.723
Deltaproteobacteria	*Desulfuromonas sp*	0.032
Synergistia	*Dethiosulfovibrio russensis*	0.214
Bacilli	*Enterococcus sp*	0.719
Clostridia	*Eubacterium sp*	0.31
Clostridia	*Geosporobacter sp*	1.435
Thermotogae	*Geotoga subterranea*	0.051
Clostridia	*Halanaerobium sp*	0.265
Gammaproteobacteria	*Halomonas sp*	0.006
Gammaproteobacteria	*Halothiobacillus sp*	0.01
Gammaproteobacteria	*Marinobacter sp*	0.058
Gammaproteobacteria	*Marinobacterium litorale*	0.019
Methanomicrobia	*Methanolobus sp*	0.01
Alphaproteobacteria	*Nesiotobacter exalbescens*	0.086
Clostridia	*Oceanirhabdus sediminicola*	0.042

Class	Species	OG150802-001
		Raw Feed
Thermotogae	*Oceanotoga sp*	1.461
Alphaproteobacteria	*Paracoccus sp*	0.022
Thermotogae	*Petrotoga mexicana*	0.182
Thermotogae	*Petrotoga sp*	0.006
Bacteroidia	*Porphyromonas canoris*	0.182
Bacteroidia	*Prolixibacter bellariivorans*	0.128
Gammaproteobacteria	*Pseudomonas balearica*	0.016
Actinobacteria	*Rhodococcus sp*	0.006
Gammaproteobacteria	*Rickettsiella sp*	0.01
Gammaproteobacteria	*Salinivibrio costicola*	0.029
Gammaproteobacteria	*Salinivibrio sp*	37.01
Sphingobacteriia	*Sphingobacterium sp*	0.016
Alphaproteobacteria	*Sphingomonas sp*	0.099
Bacilli	*Staphylococcus epidermidis*	0.061
Epsilonproteobacteria	*Sulfurimonas sp*	0.006
Synergistia	*Thermovirga sp*	0.01
Gammaproteobacteria	*Thiohalocapsa sp*	0.006
Unclassified	*Unclassified*	31.175
Gammaproteobacteria	*Vibrio harveyi*	0.006
Gammaproteobacteria	*Vibrio hepatarius*	0.058

APPENDIX A. Methods

For microbial analysis, DNA was subject to bacterial tag-encoded FLX amplicon sequencing (bTEFAP) using primers 515F- GTGCCAGCMGCCGCGGTAA and
806R- TAATCTWTGGGVHCATCAGG.

Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the XXXXXXXX primer (see above primer). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the XXXXXXX primer (see above primer). Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95○C for 5 min, then 35 cycles of 94○C for 30 sec, 54○C for 40 sec, 72○C for 1 min, followed by one cycle of 72○C for 10 min and 4○C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana). Size selected pools were then quantified and 150 ng of DNA were hybridized to OT2-400 Ion Sphere beads (Life Technologies) to create single stranded DNA following Ion PGM Protocols (Life Technologies). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing following established manufacture protocols (Life Technologies).

APPENDIX B. Overview of Select Metabolic Processes

Notes on Taxonomic and Metabolic Assignment

Organisms are referred to by the identity of the most closely matched organism in the database. However, this does not indicate 100% identity. Metabolic assignments are inferred by the metabolic characteristics of the most closely related organism for which experimental data has been provided. Some metabolic groupings are overlapping and non-exclusive, e.g. many fermentative organisms generate organic acids or are capable of sulfidogenisis under some conditions. The methods utilized for sample processing and genetic analysis are described in Appendix A. An overview of select metabolisms is provided in Appendix B.

APB: Acid-Producing Bacteria

Acid-producing bacteria are of specific interest to the oilfield community as acid production directly and aggressively promotes corrosion. Several metabolic pathways result in the production of acids, including fermentation pathways that generate organic acids such as lactic acid and acetic acid, as well as those that generate inorganic acids such as sulfuric acid as a byproduct of the oxidation of inorganic sulfur compound. It should be noted that not all fermentative pathways result in acidification of the surrounding environment. The identification of bacteria as acid producing does not necessarily indicate acidification of bulk fluids.

Biodeg: Biodegradation

Some bacterial genera and species have the capacity to utilize "atypical" or "unusual" substrates as carbon sources. These bacteria are loosely referred to as Biodeg, for "Biodegradation". The definition used here for "atypical or unusual substrates" with reference to bacterial metabolism includes compounds that most bacteria cannot utilize as a food source. Unusual compounds Biodeg organisms might be able to "eat" include disinfectants, antibiotics, xenobiotics and detergents. Some degrade long chain polymers of sugars and carbohydrates, such as those found in cell wall materials. Others are able to degrade hydrocarbons. Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons.

IRB: Iron-Reducing Bacteria, Fe(III)RB

In the absence of oxygen, many microbes can use Fe(III) as an electron acceptor, reducing it to Fe(II). Iron reduction has been observed under both acidophilic and neutrophilic conditions. Two common iron-reducing genera are Shewanella and Geobacter. In addition to IRB activity, Shewanella species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Shewanella are capable of growing in corrosive biofilms where they have been shown to remove the protective H2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. IRB should not be confused with iron oxidizing bacteria, which are aerobes responsible for a rust brown staining and slimy growth in surface waters.

NRB: Nitrate Reducing Bacteria

NRB are able to reduce nitrates to nitrites, nitrous oxide, or nitrogen under anaerobic conditions in a process termed denitrification. Most are heterotrophic facultative anaerobic bacteria

including such common bacteria as *Paracoccus, Pseudomonas, Alcaligenes,* and *Bradyrhizobioium*. A few bacteria use such reduction processes as hydrogen acceptor reactions and hence as a source of energy; in this case the end product is ammonia. Denitrification is a normal part of nitrogen cycling and not all NRB are of concern to O&G infrastructure.

A subcategory of NRB is the **NRSOB**: Nitrate-Reducing Sulfur-Oxidizing Bacteria are a specific subgroup of NRB whose levels are increased in reservoirs following nitrate injections (Gittel et al 2009; Grigoryan et al, 2008; Hubert and Voordouw, 2007). Growth of NRSOB suppresses the activity of SRB, and thus reducing sulfidogenisis. Some Epsilonproteobacteria can also oxidize petroleum sulfur compounds and utilize nitrate as an electron acceptor for growth, and thus may be considered hydrocarbon degrading. Massive dominance of related Epsilonproteobacteria has been observed in other petroleum samples, for example in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. (Kodama, Y and Kazuya Watanabe, 2003; Hubert et al, 2011). Sulfurospirillum are nitrate-reducing, sulfur oxidizing bacteria (NRSOB) members of the class Epsilonproteobacteria and are sometimes referred to as "Campylobacter" in older publications. The way in which nitrate addition can affect the SRB population involves several pathways. First, nitrate is a thermodynamically more favorable electron acceptor than sulfate, thus NRB have a competitive advantage. To emphasize the complexity of the metabolism in oilfield samples, it should be noted that under some conditions, these bacteria are also sulfidogens capable of reducing sulfur and thus producing H_2S (Finster K et al, 1997).

Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as "sulfidogens". Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks. It also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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